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(54) Title: BACTERIAL STRAINS, COMPOSITIONS INCLUDING SAME AND PROBIOTIC USE THEREOF

(57) Abstract: A biologically pure culture of an E. coli M17 bacterial strain exhibiting nalidixic acid resistance is provided. Also provided are methods of using this bacterial strains and compositions containing same.

BACTERIAL STRAINS, COMPOSITIONS INCLUDING SAME AND PROBIOTIC USE THEREOF

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to novel bacterial strains, compositions including same and methods of using such strains in probiotic treatment of gastrointestinal disorders.

Probiotics are defined as living organisms, which exert a positive effect on a host gastro-intestinal (GI) system. The most commonly used probiotics are strains of the lactic acid bacteria (LAB), particularly those classified to the *Lactobacillus*, *Lactococcus*, and *Enterococcus* genera.

It is well known that during periods of low resistance (e.g., stress or disease, at birth or following antibiotic treatments) undesirable microorganisms are able to proliferate in the gastrointestinal tract. Thus, maintaining a normal, healthy flora of microorganism in the gastrointestinal (GI) tract is critical during stressful periods.

The goal of probiotic therapy is to increase the number and activity of health-promoting microorganisms until normal GI flora can be reestablished.

Several mechanisms responsible for the protective action of probiotics have been proposed. These include, *(i)* the production of inhibitory substances (e.g., antibiotics, organic acids, hydrogen peroxide and bacteriocins) which may reduce cell viability, affect bacterial metabolism and reduce toxin production; *(ii)* blocking of adhesion sites by competitive inhibition of bacterial adhesion sites on intestinal epithelial surfaces [Conaway (1987) J. Dairy Sci. 70:1-12; Goldin (1992) Dig. Dis. Sci. 37:121-128; Kleeman and Klaenhammer (1982) J. Dairy Sci. 1982;65:2063-2069]; *(iii)* competition for nutrients; *(iv)* degradation of toxin receptors, which is the postulated mechanism by which *S. boulardii* protects animals against *C. difficile* intestinal disease through the degradation of the toxin receptor on the intestinal mucosa [Castagliuolo (1996) Infect. Immun. 64:5225-5232; Castagliuolo Infect. Immun. (1999) 67:302-307 Pothoulakis (1993) Gastroenterology 104:1108-1115]; *(v)* and stimulation of non-specific immunity [Fukushima Int. J. Food Microbiol. (1998) 42:39-44; Link-Amster FEMS Immunol. Med. Microbiol. (1994) 10:55-63; Malin Ann. Nutr. Metab. (1996) 40:137-145].

Although the effectiveness of probiotic therapy has been demonstrated by numerous studies and thus is now accepted as suitable therapy for a number of disorders, probiotic treatment can lead to a number of side effects including systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer [Marteau (2001) Safety aspects of probiotic products. Scand. J. Nutr., 45, 1, 22-24]. For example, two cases of *L. rhamnosus* infection were traced to possible probiotic consumption [Rautio (1999) Clin. Infect. Dis. 28:1159-60; Mackay (1999) Clin. Microbiol. Infect. 5:290-292]. Thirteen cases of *Saccharomyces* fungemia were caused by vascular catheter contamination [Hennequin (2000) Eur. J. Clin. Microbiol. Infect. Dis. 19:16-20] and *Bacillus* infections linked to probiotic consumption all in patients with underlying disease [Spinosa (2000) Microb. Ecol. Health Dis. 12:99-101; Oggioni (1998) J. Clin. Microbiol. 36:325-326]. Alternatively, *Enterococcus* is emerging as an important cause of nosocomial infections and isolates are increasingly vancomycin resistant.

Non-pathogenic lactose-positive E. coli comprise the main group of healthy aerobic microflora in the intestine of humans and animals, providing microbiological balance and playing an important role in alimentation and immunity.

The present inventors have previously found a single species of a non-pathogenic probiotic microorganism, designated E. coli BU-230-98, ATCC Deposit No. 202226 (DSM 12799) (E. coli M17), which is capable of restoring normal GI flora of a variety of mammals and avian. A probiotic composition comprising this probiotic organism suspended in the formulation was found to be effective in the treatment and prevention of various gastrointestinal disorders. The probiotic formulation per se was also found effective as a body weight gain enhancer and as an immuno-stimulator in mammals and avian (See U.S. Pat. Nos. 6,500,423 assigned to "The Bio Balance Corp." and related applications each of which is incorporated herein by reference).

The probiotic activities of E. coli BU-230-98, ATCC Deposit No. 202226 (DSM 12799) render it a favorable therapeutic tool for the treatment of a myriad of gastrointestinal and gastrointestinal related disorders, suggesting that assayable, antibiotic-resistant strains of this bacterial species may be of regulatory importance and used in conjunction with antibiotic treatment.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a biologically pure culture of an *E. coli* M17 bacterial strain exhibiting nalidixic acid resistance.

According to another aspect of the present invention there is provided a probiotic composition comprising, as an active ingredient, the bacterial strain and a carrier or diluent.

According to further features in preferred embodiments of the invention described below, the composition comprising 10^3-10^{10} of bacterial cells of the bacterial strain per gram of the composition.

According to still further features in the described preferred embodiments the carrier comprises a formulation for maintaining viability of the bacterial strain.

According to still further features in the described preferred embodiments the formulation comprises a volatile fraction of a plant extract.

According to still further features in the described preferred embodiments the composition further comprises an antifungal agent.

According to still further features in the described preferred embodiments the compositions further comprises an antibiotic.

According to still further features in the described preferred embodiments the composition further comprises a probiotic microorganism selected from the group consisting of a yeast cell, a mold and a bacterial cell.

According to still further features in the described preferred embodiments the carrier is a colonization carrier.

According to yet another aspect of the present invention there is provided a food additive comprising as an active ingredient, the bacterial strain and a carrier suitable for human consumption.

According to still further features in the described preferred embodiments the colonization carrier is selected from the group consisting of a saccharide, a modified saccharide and a combination thereof.

According to still another aspect of the present invention there is provided a feed additive comprising as an active ingredient, the bacterial strain and a carrier suitable for animal consumption.

According to still further features in the described preferred embodiments the carrier is selected from the group consisting of limestone, saccharides and wheat midds.

According to an additional aspect of the present invention there is provided a foodstuff comprising the bacterial strain.

According to still further features in the described preferred embodiments the foodstuff is a milk product.

According to yet an additional aspect of the present invention there is provided a method of treating a gastrointestinal disorder, the method comprising administering to a subject in need thereof a therapeutically effective amount of the bacterial strain, thereby treating the gastrointestinal disorder.

According to still further features in the described preferred embodiments the gastrointestinal disorder is selected from the group consisting of pouchitis, ulcerative colitis, Crohn's disease, inflammatory bowel disease, celiac disease, small bowel bacterial overgrowth, gastroesophageal reflux disease, diarrhea, *Clostridium difficile* colitis and/or antibiotic associated diarrhea, irritable bowel syndrome, irritiable pouch syndrome, acute diarrhea, traveller's diarrhea, lactose intolerance, HIV-associated diarrhea, sucrose isomaltase deficiency, carcinogenesis, enteral feeding associated diarrhea, and disorders which are associated with enteropathogens, non-erosive esophageal reflux disease (NERD) and associated small bowel bacterial overgrowth, functional dyspesia, necrotizing enterocolitis, diabetes gastropathy and constipation.

According to still further features in the described preferred embodiments the bacterial strain comprises all the identifying characteristics of ATCC Deposit No. PTA-7295.

According to still further features in the described preferred embodiments the E. coli M17 is ATCC Deposit No. 202226 (DSM 12799).

According to still further features in the described preferred embodiments the E. coli M17 is selected from the group consisting of BU-239, BU-230-98, BU230-01 and ATCC Deposit No. 202226 (DSM 12799).

According to still further features in the described preferred embodiments the bacterial strain comprises a genomic nucleic acid sequence selected from the group consisting of SEQ ID NO: 1-9.

According to still further features in the described preferred embodiments the bacterial strain is capable of proliferating and colonizing in a mammalian gastrointestinal tract.

According to still an additional aspect of the present invention there is provided a method of detecting presence of the bacterial strain in a fecal sample, the method comprising detecting bacterial growth in the presence of nalidixic acid, thereby detecting presence of the bacterial strain in the fecal sample.

According to a further aspect of the present invention there is provided a biologically pure culture of an *E. coli* having all identifying characteristics of ATCC Deposit No. PTA-7295.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel bacterial strains, compositions including same and probiotic use thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 shows Pulsed Field Gel Electrophoresis of the M17 parental strain and the nalidixic acid-resistant ($M17_{SNAR}$) derivatives generated according to the teachings of the present invention. Each strain was digested with XbaI. The Markers consist of lambda concatamers. MC1061, which is a K-12 derivative of *E. coli* and is genetically unrelated to M17, was run as a negative control. The strains are indicated above the relevant lane of the image.

FIG. 2 shows Amplified Fragment Length Polymorphism (AFLP) analyses of the M17 parental and $M17_{SNAR}$ strains. DNA from M17, $M17_{SNAR}$, and ECOR strains was subjected to AFLP analysis using the EcoRI-A + MseI GA primer combination. The reaction products were resolved by denaturing gel polyarcylamide gel electrophoresis on a Li-Cor/NEN 4200 global analyzer. The strain is indicated above the appropriate lane containing the resolved reaction products.

FIGs. 3a-d are graphs showing the shedding of total coliforms and $M17_{SNAR}$ in fecal samples of canines dosed with $M17_{SNAR}$. Animals [dogs 3028 (Figure 3a) and 3029 (Figure 3b) were male; dogs 3032 (Figure 3c) and 3033 (Figure 3d) were female] were dosed with 1×10^{13} colony forming units of $M17_{SNAR}$ on fourteen consecutive days after day 0 (the high dose intake in the Ricerca study). Fecal samples were collected and total coliforms enumerated in duplicate on VRBA media while $M17_{SNAR}$ was enumerated on VRBA + 25 μg/ml nalidixic acid. Nalidixic acid-

resistant colonies were confirmed with the Contig 127 PCR assay. Results are shown separately for each animal.

FIG. 4 is a scheme showing whole genome shotgun sequencing approach for *E. coli* $M17_{SNAR}$.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of bacterial strains, which can be used in the treatment of gastrointestinal and immune-related disorders.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

The present inventors have previously found a single species of a non-pathogenic probiotic microorganism, *E. coli* BU-230-98, ATCC Deposit No. 202226 (DSM 12799), which is capable of restoring normal GI flora of a variety of mammals and avian. A probiotic composition comprising this probiotic organism was found to be effective in the treatment and prevention of various gastrointestinal disorders. The probiotic composition was also found effective as a body weight gain enhancer and as an immuno-stimulator in mammals and avian (See U.S. Pat. Nos. 6,500,423 assigned to "The Bio Balance Corp." and related applications each of which is incorporated herein by reference).

The probiotic activities of E. coli BU-230-98, ATCC Deposit No. 202226 (DSM 12799) (M17) render it a favorable therapeutic tool for the treatment of a myriad of gastrointestinal disorders and related disorders (e.g., immune related), indicating that antibiotic resistant strains of this bacterial species may be of regulatory importance and used in combination with antibiotic treatment.

While reducing the present invention to practice the present inventors have isolated, through laborious experimentations, spontaneously occurring nalidixic acid-

resistant mutant derivatives of E. coli BU-230-98, ATCC Deposit No. 202226 (DSM 12799), termed $M17_{SNAR}$ and developed a method for specific enumeration and confirmation of this strain in fecal samples.

As illustrated hereinbelow and in the Examples section which follows, the present inventors have enriched and selected from a parental stock of E. coli BU-230-98, ATCC Deposit No. 202226 (DSM 12799), naturally-occurring, nalidixic acid-resistant mutants. The nalidixic acid-resistant strain termed $M17_{SNAR}$ ATCC Deposit No. 7295, is indistinguishable from the parental strain by PFGE and AFLP analyses. Unique nucleic acid sequences of the $M17_{SNAR}$ genome were then identified by whole genome sequencing and computational analysis followed by PCR analysis against a panel of *E. coli* strains. Introduction of the $M17_{SNAR}$ into composite fecal samples, followed by selective plating on VRBA and confirmation by nucleic acid analysis showed that the $M17_{SNAR}$ could be specifically quantified in spiked fecal samples. The limit of detection of the assay was estimated to be 33 CFU/ml of $M17_{SNAR}$ in a fecal sample. Given the combination of selective plating on VRBA + nalidixic acid and the confirmation by nucleic acid analysis, the assay appears to be highly specific for detecting and quantifying *E. coli* $M17_{SNAR}$ in fecal samples.

Thus, according to one aspect of the present invention there is provided a biologically pure culture of an *E. coli* M17 bacterial strain exhibiting nalidixic acid resistance (see Example 1 of the Examples section which follows).

As used herein a " *E. coli* M17 bacterial strain" refers to the strain *per se* and non-pathogenic derived strains which maintain a probiotic activity and biochemical characteristics as listed in Tables 1-3, below.

"Probiotic activity" as used herein refers to the property of inhibiting the growth of at least one pathogen. Testing the inhibition of pathogen growth may be effected on solid medium in which culture supernatants of candidate isolated bacteria are observed for their property of inhibiting the growth of a pathogen when applied to the surface of the solid medium. Typically, a paper disc impregnated with the culture supernatant of a candidate probiotic strain is placed on the surface of an agar plate seeded with the pathogen. Probiotic bacterial supernatants cause a ring of clear agar or of reduce growth density indicating inhibition of the pathogen in the vicinity of the disc. There are other tests for inhibition which are available or could be devised,

including direct growth competition tests, in vitro or in vivo which can generate a panel of probiotic bacteria similar to that described herein. The bacterial strains identified by any such test are within the category of probiotic bacteria, as the term is used herein.

Examples of such strains and in vitro characteristics of same are provided in Tables 1-3 below. According to a preferred embodiment of this aspect of the present invention, the *E. coli* M17 bacterial strain is BU-239, BU-230-98, BU-230-01 and ATCC Deposit No. 202226 (DSM 12799).

Table 1 - In Vitro Characterization Studies: Various E. coli Probiotic Strains

Strain/Code	BU 239 (original M-17)	BU 230-98 (BioBalance, M-17 Industrial Stock)	BU 230-01 (BioBalance, M-17 Industrial Stock)
Serotype	O2	O2	O2
Physical Characterization	Gram negative rods	Gram negative rods	Gram negative rods
Metabolic Characterization	Ferments glucose Reduces nitrates to nitrites Oxidase neg. Catalase pos.	Ferments glucose Reduces nitrates to nitrites Oxidase neg. Catalase pos.	Ferments glucose Reduces nitrates to nitrites Oxidase neg. Catalase pos.

Table 2 - Fermentation Profile for Various E. coli strain M-17 Samples using API 20E

Fermentation Substrate	BU-239 (Original *E. coli* strain M-17)	*E. coli* strain *M-17*, ATCC 202226 (DSM 12799) (BioBalance Deposited Master Seed Stock)	*E. coli* strain *M-17* (Tarasevich Institute, Moscow, Official Sample)
Ortho-nitrophenyl-beta-D-galactopyranoside	+	+	+
Arginine dihydrolase	-	-	-
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Citrate	-	-	-
H_2S	-	-	-
Urease	-	-	-
Tryptophan deaminase	-	-	-
Indole	+	+	+
Voges-Proskauer	-	-	-
Gelatin	-	-	-
Glucose	+	+	+
Mannitol	+	+	+
Inositiol	-	-	-

Fermentation Substrate	BU-239 (Original *E. coli* strain M-17)	*E. coli* strain *M-17*, ATCC 202226 (DSM 12799) (BioBalance Deposited Master Seed Stock)	*E. coli* strain *M-17* (Tarasevich Institute, Moscow, Official Sample)
Sorbitol	+	+	+
Rhamnose	+	+	+
Sucrose	+	+	+
Melibiose	+	+	+
Amygdalin	-	-	-
Arabinose	+	+	+

Table 3 - In Vitro Characterization Studies: Presence of Virulence Factors in E. coli Strain M-17 as Detected by PCR

Category of Pathogenic *E. coli*	Type of Virulence Factor(s)	Virulence Factor Designation(s)	*E. coli* Strain M-17 Isolate		
			BU-239 (original)	ATCC 202226 (DSM 12799)	Tarasevich (Russian)
Uropathogenic	Adhesion factors	Type I (Fim A) AFA SFA	+ - -	+ - -	+ - -
Uropathogenic – septicemic	Adhesion factors (P fimbriae)	PapC PapG	- -	- -	- -
Uropathogenic – septicemic – meningitis assoc.	Aerobactin	iuc	-	-	-
Enterohemorragic – uropathogenic	Hemolysins	HlyA, HlyC Ehx	- -	- -	- -
Enterohemorragic – enteropathogenic	Attaching and effacing gene	pas	-	-	-
	Intimin	eae	-	-	-
Enterohemorragic	Shigatoxins	Stx1, Stx2 VT2vpl, VT2vh SLT I, SLT II	- -	- -	- -
	Flagellar antigen	FliC	-	-	-
	O serogroup	O157	-	-	-
	H serotype	H7	-	-	-
Enteropathogenic	Attaching and effacing factor	EAE	-	-	-
	Bundle forming pili	bfp	-	-	-
Enteroaggregative	Adhesion factors	aggR AAF/I	- -	- -	- -
	Toxin	EAST1	-	-	-
Enterotoxigenic	Adhesion factors	CFA1, CFA2 (CS1coo) CFA2 (CS3 cst)	- - -	- - -	- - -
	Adhesion	F4 (K88)	-	-	-

Category of Pathogenic *E. coli*	Type of Virulence Factor(s)	Virulence Factor Designation(s)	*E. coli* Strain M-17 Isolate		
			BU-239 (original)	ATCC 202226 (DSM 12799)	Tarasevich (Russian)
	factors (shared by porcine and bovine	F5 (K99)	-	-	-
		F18	-	-	-
		F41	-	-	-
	Enterotoxins	LT, StaH	-	-	-
		STaP, STb	-	-	-
Extraintestinal	Adhesion factor	CS31a	-	-	-
	Autotransporter	Tsh	-	-	-

As mentioned the E. coli BU-239 bacterial strain of the present invention exhibits nalidixic acid resistance.

As used herein the phrase "nalidixic acid resistance" refers to the ability of the culture of this aspect of the present invention to multiply even in the presence of the quinolone antibiotic, nalidixic acid (NegGram). Cultures of the present invention are preferably resistant to at least 5 μg/ml nalidixic acid, more preferably at least 15 μg/ml nalidixic acid, even more preferably 25 μg/ml nalidixic acid, even more preferably 50 μg/ml nalidixic acid, even more preferably 100 μg/ml nalidixic acid.

The isolation, identification and culturing of the bacterial strains of the present invention (i.e., nalidixic acid resistant BU-239 strains) can be effected using standard microbiological techniques. Examples of such techniques may be found in Gerhardt, P. (ed.) Methods for General and Molecular Microbiology. American Society for Microbiology, Washington, D.C. (1994) and Lennette, E. H. (ed.) Manual of Clinical Microbiology, Third Edition. American Society for Microbiology, Washington, D.C. (1980).

Isolation of the bacterial strains of the present invention is preferably effected by streaking the specimen (e.g., E. coli BU-230-98) on a solid medium (e.g., nutrient agar plates) to obtain a single colony which is characterized by the phenotypic traits described hereinabove (e.g., Gram negative, capable of lactose fermentation and nalidixic acid resistance) and to reduce the likelihood of working with a culture which has become contaminated and/or has accumulated mutations.

The bacterial strains of the present invention can be propagated in a liquid medium under conditions which are described in the Examples section.

Medium for growing the bacterial strains of the present invention includes a carbon source, a nitrogen source and inorganic salts as well as specially required substances such as vitamins, amino acids, nucleic acids and the like (Examples 1 of the Examples section which follows describes embodiments of medium compositions which can be used in accordance with the present invention).

Examples of suitable carbon sources which can be used for growing the bacterial strains of the present invention include, but are not limited to, starch, peptone, yeast extract, amino acids, sugars such as glucose, arabinose, mannose, glucosamine, maltose, and the like; salts of organic acids such as acetic acid, fumaric acid, adipic acid, propionic acid, citric acid, gluconic acid, malic acid, pyruvic acid, malonic acid and the like; alcohols such as ethanol and glycerol and the like; oil or fat such as soybean oil, rice bran oil, olive oil, corn oil, sesame oil. The amount of the carbon source added varies according to the kind of carbon source and is typically between 1 to 100 gram per liter medium. Preferably, glucose, starch, and/or peptone is contained in the medium as a major carbon source, at a concentration of 0.1-5% (W/V).

Examples of suitable nitrogen sources which can be used for growing the bacterial strains of the present invention include, but are not limited to, amino acids, yeast extract, tryptone, beef extract, peptone, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate, ammonium phosphate, ammonia or combinations thereof. The amount of nitrogen source varies according the nitrogen source, typically between 0.1 to 30 gram per liter medium.

As the inorganic salts, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, magnesium sulfate, magnesium chloride, ferric sulfate, ferrous sulfate, ferric chloride, ferrous chloride, manganous sulfate, manganous chloride, zinc sulfate, zinc chloride, cupric sulfate, calcium chloride, sodium chloride, calcium carbonate, sodium carbonate can be used alone or in combination. The amount of inorganic acid varies according to the kind of the inorganic salt, typically between 0.001 to 10 gram per liter medium.

Examples of specially required substances include, but are not limited to, vitamins, nucleic acids, yeast extract, peptone, meat extract, malt extract, dried yeast and combinations thereof.

Cultivation is effected at a temperature, which allows the growth of the probiotic bacterial strains of the present invention, essentially, between 28 °C and 46 °C. A preferred temperature range is 30-37 °C.

For optimal growth, the medium is preferably adjusted to pH 7.0 – 7.4.

It will be appreciated that commercially available media may also be used to culture the bacterial strains of the present invention, such as Luria Broth available from Difco, Detroit, MI.

Bacterial cells thus obtained are isolated using methods, which are well known in the art. Examples include, but are not limited to, membrane filtration and centrifugal separation.

The pH may be adjusted using sodium hydroxide and the like and the culture may be air dried or dried using a freeze dryer, until the water content becomes equal to 4 % or less.

Once a lot of the bacterial strains of the present invention is generated, it is preferably quality qualified. Such qualification may include testing resistance to nalidixic acid, lactose fermentation, resistance to gastric acidity, gastrointestinal tract colonization, resistance to bile acid, which correlates with gastric survival *in vivo*, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, ability to reduce pathogen adhesion to surfaces and bile salt hydrolase activity [Conway (1987) J. Dairy Sci. 70:1-12].

Newly isolated strains are preferably further characterized as being molecularly indistinguishable from the parental strain while still exhibiting nalidixic acid resistance. This may be attributed to the presence of genomic sequences such as set forth in SEQ ID NO: 1-9 or homologous sequences (e.g., above about 80 %, 90 % or 95 % identity).

Using the above methodology, the present inventors were able to isolate the strains of the present invention, displaying varying levels of antibiotic resistance as listed in Table 7 below.

According to a preferred embodiment of this aspect of the present invention the strain has all identifying characteristics of the strain deposited under the Budapest Treaty in the American Type Culture Collection (ATCC) on December 22, 2005, as strain PTA – 7295 (referred to herein as $M17_{SNAR}$).

The probiotic bacterial strains of the present invention exhibit antibiotic resistance and as such may be adventitiously used, from a regulatory point of view, for the treatment of a variety of gastrointestinal disorders, simply because tracking of same in fecal samples is now allowed. Additionally, co-treatment with the strains of the present invention with nalidixic acid is now allowed, thus maintaining a viable gastrointestinal flora while practicing antibiotic treatment such as for urinary tract infection.

Thus, according to still another aspect of the present invention there is provided a method of treating or preventing a gastrointestinal disorder in a subject.

The method is effected by administering to a subject in need thereof a therapeutically effective amount of the probiotic bacterial strains of the present invention.

As used herein the term "treating" refers to alleviating or diminishing a symptom associated with a disorder. Preferably, treating cures, e.g., substantially eliminates, the symptoms associated with the disorder.

Subjects which may be treated with the bacterial cultures of the present invention include humans and animals which may benefit from probiotic treatment. Examples include but are not limited to mammals, reptiles, birds, fish and the like.

Examples of gastrointestinal disorders which may be treated using the probiotic strains of the present invention include, but are not limited to, pouchitis (e.g., associated with ileal pouch-anal anastomosis post ulcerative colitis), ulcerative colitis, Crohn's disease, inflammatory bowel disease, celiac disease and associated small bowel bacterial overgrowth, gastroesophageal reflux disease and associated small bowel bacterial overgrowth, small bowel bacterial overgrowth, diarrhea and high stool output in patients with ileostomy, antibiotic-associated diarrhea, *Clostridium difficile* colitis and/or antibiotic associated diarrhea, irritable bowel syndrome, irritiable pouch syndrome, acute diarrhea, traveller's diarrhea, lactose intolerance, HIV-associated diarrhea, sucrose isomaltase deficiency, carcinogenesis, enteral feeding associated diarrhea, and disorders which are associated with enteropathogens such as *Helicobacter pylori, Campylobacter jejuni, Campylobacter coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Enterococcus faecalis, Haemophilus influenzae,*

Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Citrobacter freundii, Serratia marcescens, Pseudomonas aeruginosa and *Pseudomonas maltophilia, Salmonella sp.* Viruses such as rotavirus and fungi such as *Candida albicans* and *Aspergillus fumigatus*, and combinations of these species.

Additional disorders which may be treated using the strains of the present invention include, but are not limited to, non-erosive esophageal reflux disease (NERD) and associated small bowel bacterial overgrowth, functional dyspesia, necrotizing enterocolitis, diabetes gastropathy, constipation (associated with the changes in gastrointestinal microflora), uro-genital tract associated diseases, urinary bladder infections (uterine infections and infections of the cervix, vagina and vulva commonly occur in human beings and domestic animals, especially following birth). Typical infecting organisms of the endometrium (i.e., uterine mucosa) and contiguous mucosal surfaces in the lower genital tract include, for example, β-hemolytic streptococci, *Candida albicans, Klebsiella pneumoniae*, coliform bacteria including *Escherichia coli, Corynebacterium pyogenes* and *C. vaginale*, various *Campylobacter* or *Trichomonas* species such as *T. vaginalis*, and the like (see U.S. Pat. No. 5,667,817). Other urogenital pathogens include but are not limited to *Chlamydia trachomatis, Neisseria gonorrhoeae,* herpes simplex virus, HIV, papillomavirus and *Treponema pallidum.*], respiratory diseases associated with pathogenic bacteria including but not limited to, *Staphylococcus aureus, Streptococcus pneumoniae,* beta-hemolytic streptococci and *Haemophilus influenza*; rheumatoid arthritis [Malin (1996) Br J Rheumatol;35:689–94], allergies associated with reduced microbial stimulation associated with the western world lifestyle (i.e., improvement of hygiene and reduced family size). Atopic diseases such as associated with the decrease in *Lactobacillus* and *Eubacterium* combined with higher counts of *Clostridium ssp* [Biorksten et al. Clin. Exp. Allergy (1999) 29:342-346].

It will be appreciated that the bacterial strains of the present invention may be used to treat other diseases or disorders (i.e., extraintestinal), which may be treated by probiotics.

The ability of the bacterial strains of the present invention to treat bacterial, fungal or viral infections in other organs is an outcome of stimulating multiple defense mechanisms [reviewed by Isolauri (2001) Am. J. Clin. Nut. 73:444S-450S]

including promotion of a nonimmunologic gut defense barrier which may inhibit translocation of potential pathogens and thus prevent infections of the blood stream and other tissues or organs. Another defense mechanism is improvement of the intestine's immunologic barrier, particularly through intestinal immunoglobulins A responses and alleviation of intestinal inflammatory responses which produce a gut stabilizing effect. As well as by immune regulation, particularly through balance control of proinflammatory and anti-inflammatory cytokines.

Examples of extraintestinal diseases which can be treated with the probiotic cultures of the present invention include, but are not limited to appendicitis, autoimmune disorders, multiple sclerosis, rheumatoid arthritis, coeliac disease, small bowel or gastric overgrowth associated with diabetic gastropathy, organ transplantation, periodontal disease, urogenital diseases (vaginal, urethral and perineal), surgical associated trauma, surgical-induced metastatic disease, sepsis, weight loss, anorexia, fever control, cachexia, wound healing, ulcers, gut barrier function, allergy, asthma, respiratory disorders, rhinovirus-associated diseases (e.g., otitis media, sinusitis, asthma and pulmonary diseases), hepatic diseases (e.g., hepatic encephalopathy) constipation, nutritional disorders, epidermal disorders, psoriasis, anthrax and/or acne vulgaris [see Examples 5-8, U.S. Pat. Application No. 20030113306, Rolfe (2000) Journal of Nutrition 130:396S-402S and Background section].

Typical concentration range of probiotic microorganisms administered, according to this aspect of the present invention, is 10^3 to 10^{13} cells per day. Preferably, at least about 10^6, at least about 10^7, at least about 10^{10} cells per day are used in probiotic administration (see U.S. Pat. Nos. 6,221,350 and 6,410,305). However, it will be appreciated that the amount of bacteria to be administered will vary according to a number of parameters including subject's size, type of disorder and severity of symptoms.

The bacterial cultures of the present invention can be formulated in a nutritional composition (e.g., foodstuff, food additive or feed additive). For example, the bacterial strains of the present invention may be included in fermented milk products (i.e., nutraceuticals), such as described in U.S. Pat. No. 6,156,320.

Alternatively, the bacterial strain of the present invention may be formulated in a pharmaceutical composition, where it is mixed with a pharmaceutically acceptable carrier preferably for oral or enteral administration route, selected according to the intended use.

Herein the term "active ingredient" refers to the bacterial preparation accountable for the biological effect.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases. One of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

In addition to carriers the pharmaceutical compositions or nutritional compositions of the present invention may also include, colonization carriers, formulations for maintaining viability of the bacterial strain (e.g., volatile fraction of a plant extract, see U.S. Pat. No. 6,500,423), nutrients, antibiotics, anti-fungal agents, antioxidants, plant extracts, buffering agents, coloring agents, flavorings, vitamins and

minerals, which are selected according to the intended use and the route of administration employed.

Colonization carriers – The compositions of the present invention may include a colonization carrier which transports the probiotic microorganisms to the large bowel or other regions of the gastrointestinal tract. Typically the carrier is a saccharide such as amylose, inulin, pectin, guar gum, chitosan, dextrans, cyclodextrins, alginate and chondroitin sulphate [Chourasia and Jain (2003) J. Pharm. Pharmaceut. Sci. 6:33-66].

Preferably, modified and/or unmodified resistant starches are used as colonization carriers (see U.S. Pat. No. 6,221,350).

The phrase "resistant starch" refers to starch forms defined as RS1, RS2, RS3 and RS4 as defined in Brown, McNaught and Moloney (1995) Food Australia 47: 272-275. Typically, a resistant starch is used in a probiotic composition since it is essentially not degraded until it reaches the large bowel. Therefore it provides a readily available substrate for fermentation by the probiotic microorganisms once they reach the large bowel. Preferably, the resistant starch is a high amylose starch, including but not limited to maize starch having an amylose content of 50 % w/w or more, particularly 80 % w/w or more, rice and wheat starch having an amylose content of 27 % w/w or more and; particular granular size ranges of starches having an amylose content of 50 % or more and enhanced resistant starch content, these starches including maize, barley, wheat and legumes. Other forms of resistant starch derived from sources such as bananas or other fruit types, tubers such as potatoes, and mixtures or combinations thereof can also be used in accordance with the present invention.

It will be appreciated that it may be advantageous to chemically modify the starch, such as by altering the charge, density or hydrophobicity of the granule and/or granule surface to enhance the attachment compatibility between the microorganism and the resistant starch. Chemical modifications, such as etherification, esterification, acidification and the like are well known in the art and may be utilized to modify the starch. Alternatively, modifications can be induced physically or enzymically such as described in U.S. Pat. No. 6,221,350.

The colonizing carrier may also be an oligosaccharide. Oligosaccharides are known to increase the number of probiotic microorganisms in the gastrointestinal tract. Examples of commercially available oligosaccharides which can be used as colonizing carriers include but are not limited fructo-, galacto-, malto-, isomalto-, gentio-, xylo-, palatinose-, soybean- (including raffinose and stachyose), chito-, agaro-, neoagaro-, α-gluco-, β-gluco-, cyclo-inulo-, glycosylsucrose, lactulose, lactosucrose and xylsucrose.

The oligosaccharide can be used in the composition in a concentration of about 0.01 to 10 % (w/w). Preferably the concentration of the oligosaccharide is about 0.05 to 5 %.

Preferably, a combination of starch and an oligosaccharide is used as the colonizing agent of this aspect of the present invention.

Antibiotics – The compositions of the present invention may include nalidixic acid preferably at a range selected from 0.1-10 μg/mL.

Anti-fungal agents - The compositions of the present invention may include a therapeutically-effective amount of an anti-fungal agent. Typical anti-fungal agents which may be utilized include, but are not limited to: Clotrimazole, Fluconazole, Itraconazole, Ketoconazole, Miconazole, Nystatin, Terbinafine, Terconazole, Tioconazole, and the like.

Antioxidants, buffering agents, plant extracts, coloring agents, flavorings, vitamins and minerals - The compositions of the present invention may include antioxidants, buffering agents, plant extracts and other agents such as coloring agents, flavorings, vitamins or minerals. For example, the composition of the present invention may contain one or more of the following minerals: calcium citrate (15-350 mg); potassium gluconate (5-150 mg); magnesium citrate (5-15 mg); and chromium picollinate (5-200 μg). In addition, a variety of salts may be utilized, including calcium citrate, potassium gluconate, magnesium citrate and chromium picollinate. Chemicals are commercially available from Spectrum Quality Products, Inc (Gardena, Calif.), Sigma Chemicals (St. Louis, Mo.), Seltzer Chemicals, Inc., (Carlsbad, Calif.) and Jarchem Industries, Inc., (Newark, N.J.). Examples of plant extracts, which can be used in accordance with the present invention include but are not limited to chamomile, bur-marigold, St. John's wort, ginger and other approved plant extracts

which are FDA approved [for review see O'Hara M, Kiefer D, Farrell K, Kemper K. Arch Fam Med. (1998) Nov-Dec;7(6):523-36.; Modesto A, Lima KC, de Uzeda M. ASDC J Dent Child. (2000) Sep-Oct;67(5):338-44,302; Lee KG, Shibamoto T. J Agric Food Chem. (2002) Aug 14;50(17):4947-52].

Thickening agents - Thickening agents may be added to the compositions such as polyvinylpyrrolidone, polyethylene glycol or carboxymethylcellulose.

Carriers - The active agents (e.g., bacterial cells) of the compositions of the present invention are combined with a carrier, which is physiologically compatible with the tissue of the species to which it is administered (i.e., suitable for human consumption or animal consumption). The carriers, according to this aspect of the present invention can be solid-based, dry materials for formulation into tablet, capsule or powdered form. Alternatively, the carrier can be of liquid or gel-based materials for formulations into liquid or gel forms. The specific type of carrier, as well as the final formulation depends, in part, upon the selected route(s) of administration.

Typical carriers for dry formulations include, but are not limited to: alginate (e.g., calcium alginate), trehalose, malto-dextrin, rice flour, micro-crystalline cellulose (MCC), magnesium stearate, inositol, fructo-oligosaccharides (FOS), gluco--oligosaccharide (GOS), dextrose, sucrose, and the like. Where the composition is dry and includes evaporated oils that may cause the composition to cake (i.e., adherence of the component spores, salts, powders and oils), it is preferred to include dry fillers, which distribute the components and prevent caking. Exemplary anti-caking agents include MCC, talc, diatomaceous earth, amorphous silica, gelatin, saccharose, skimmed dry milk powder, starch and the like, which are typically added in an amount of from approximately 1% to 95% by weight. It will be appreciated that dry formulations, which are subsequently rehydrated (e.g., liquid formula) or given in the dry state (e.g., chewable wafers, pellets or tablets) are preferred to initially hydrated formulations. Dry formulations (e.g., powders) may be added to supplement commercially available foods (e.g., liquid formulas, strained foods, or drinking water supplies).

Suitable liquid or gel-based carriers include but are not limited to: water and physiological salt solutions; urea; alcohols and derivatives (e.g., methanol, ethanol,

propanol, butanol); glycols (e.g., ethylene glycol, propylene glycol, and the like). Preferably, water-based carriers have a neutral pH value (i.e., pH 7.0).

Preservatives may also be included within the carrier including methylparaben, propylparaben, benzyl alcohol and ethylene diamine tetraacetate salts. The compositions of the present invention may also include a plasticizer such as glycerol or polyethylene glycol (with a preferred molecular weight of MW=800 to 20,000). The composition of the carrier can be varied so long as it does not interfere significantly with the pharmacological activity of the active ingredients or the viability of the bacterial strains of the present invention. Other types of carriers, which can be used according to this aspect of the present invention are described hereinbelow.

Nutrient supplements - A nutrient supplement component of the compositions of the present invention can include any of a variety of nutritional agents, which are well known in the art, including vitamins, minerals, essential and non-essential amino acids, carbohydrates, lipids, foodstuffs, dietary supplements, and the like. Thus, the compositions of the present invention can include fiber, enzymes and other nutrients. Preferred fibers include, but are not limited to: psyllium, rice bran, oat bran, corn bran, wheat bran, fruit fiber and the like. Dietary or supplementary enzymes such as lactase, amylase, glucanase, catalase and the like can also be included. Vitamins for use in the compositions of the present invention include vitamins B, C, D, E, folic acid, K, niacin, and the like. Typical vitamins are those, recommended for daily consumption and in the recommended daily amount (RDA).

The pharmaceutical composition of the present invention is formulated according to the intended use. A review of conventional formulation techniques can be found in e.g. "The Theory and Practice of Industrial Pharmacy" (Ed. Lachman L. et al, 1986) or Laulund (1994).

Preferably, the compositions of the present invention are formulated for enteral administration.

As used herein the phrase "enteral administration" refers to administration of a pharmacological agent through any part of the gastro-intestinal tract, such as rectal administration, colonic administration, intestinal administration (proximal or distal) and gastric administration.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as alginate (e.g., calcium alginate), sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

It will be appreciated that the compositions of the present invention can be encapsulated into an enterically-coated, time-released capsule or tablet. The enteric

coating allows the capsule/tablet to remain intact (i.e., undissolved) as it passes through the gastrointestinal tract, until such time as it reaches the small intestine.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

A number of examples for parenteral administration of live bacteria cells are known in the art [see for example, Tjuvajev (2001) J. Control Release 74(1-3):313-5. Rosenberg (2002) J. Immunother. 25:218-25; Sheil (2004) Gut 53(5):694-700; and Matsuzaki (2000) Immunol. Cell Biol. 78(1):67-73]. It will be appreciated that bacteria cells of the present invention may also be administered in an attenuated form so as to modulate immune responses [Matsuzaki (2000) Immunol. Cell Biol. 78(1):67-73].

The preparation of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Formulations suitable for genital application include solutions.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, jelly, foams or sprays or aqueous or oily suspensions, solutions or emulsions (*i.e.*, liquid formulations), or films containing carriers as are known in the art to be appropriate (described in details in U.S. Pat. No. 5,756,681).

Compositions suitable for application to the vagina are disclosed in U.S. Pat. NOs: 2,149,240, 2,330,846, 2,436,184, 2,467,884, 2,541,103, 2,623,839, 2,623,841, 3,062,715, 3,067,743, 3,108,043, 3,174,900, 3,244,589, 4,093,730, 4,187,286, 4,283,325, 4,321,277, 4,368,186, 4,371,518, 4,389,330, 4,415,585, 4,551,148, 4,999,342, 5,013,544, 5,227,160, 5,229,423, 5,314,917, 5,380,523, and 5,387,611.

For transurethral administration the composition contains one or more selected carriers excipients, such as water, silicone, waxes, petroleum jelly, polyethylene glycol (PEG), propylene glycol (PG), liposomes, sugars such as mannitol and lactose,

and/or a variety of other materials, with polyethylene glycol and derivatives thereof. It is preferred that the pharmaceutical compositions contain one or more transurethral permeation enhancers, *i.e.*, compounds which act to increase the rate at which the selected drug permeates through the urethral membrane. Examples of suitable permeation enhancers include dimethylsulfoxide (DMSO), dimethyl formamide (DMF), N,N-dimethylacetamide (DMA), decylmethylsulfoxide, polyethylene glycol monolaurate (PEGML), glycerol monolaurate, lecithin, the 1-substituted azacycloheptan-2-ones, particularly 1-n-dodecylcyclaza-cycloheptan-2-one (available under the trademark Azone^{RTM} from Nelson Research & Development Co., Irvine, Calif.), SEPA^{RTM} (available from Macrochem Co., Lexington, Mass.), alcohols (e.g., ethanol), surfactants including, for example, Tergitol^{RTM}, Nonoxynol-9^{RTM} and TWEEN-80^{RTM}, and lower alkanols such as ethanol. As disclosed in WO91/16021, transurethral administration of an agent can be carried out in a number of different ways. For example, the agent can be introduced into the urethra from a flexible tube, squeeze bottle, pump or aerosol spray. The agent may also be contained in coatings, pellets or suppositories, which are absorbed, melted or bioeroded in the urethra. In certain embodiments, the agent is included in a coating on the exterior surface of a penile insert.

Formulations of the present invention are selected so as to maintain bacterial viability. However, when the use of attenuated bacteria is desired, formulations of the present invention may be selected of a broader range.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

Typically bacteria species of the present invention (i.e., active ingredient) may constitute 1-90 %, more preferably 5-90 %, even more preferably 10-90 % by weight of the final composition and still more preferably 15-88% % by weight contained within a formulation suitable for administration. Alternatively , the composition of

the present invention may contain at least 10^6, more preferably at least 10^8, even more preferably at least 10^{10} viable bacteria per one dose of composition.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals (see Examples 1-4 of the Examples section which follows). The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

To ensure that bacteria of the present invention are able to withstand conditions of product manufacture (e.g., food industry hazardous conditions), product (either pharmaceutical compositions, food additives, feed) shelf life and transit through the gastro-intestinal tract, bacterial cells are preferably encapsulated.

Methods of encapsulating live bacterial cells are well known in the art (see e.g., U.S. Patent to General Mills Inc. such as U.S. Pat. No. 6,723,358). For example, micro-encapsulation with alginate and and Hi-MaizeTM starch followed by freeze-drying has been proved successful in prolonging shelf-life of bacterial cells in dairy products [see e.g., Kailasapathy et al. Curr Issues Intest Microbiol. 2002 Sep;3(2):39-48]. Alternatively, entrapment of viable probiotic in sesame oil emulsions may also be used [see e.g., Hou et al. J. Dairy Sci. 86:424-428].

As mentioned hereinabove, the probiotic compositions of the present invention can be provided to animals using methods, which are well known in the art.

Typically, the probiotic composition is introduced into the animal's gastrointestinal tract via a feed additive, which is added to a feed diet. Alternative methods of administration are liquid ingestion, paste or gel ingestion, boles, powder dusting surface of animal and the like.

In addition to probiotic bacterial cells, the feed additive may include, for example, carrier materials such as, limestone and wheat midds (see U.S. Pat. No. 6,410,305). The feed additive can be added to the animal's regular diet at a rate of 0.01 to 10 and preferably about 0.5 to 2.5 pounds of additive per ton of animal feed.

The feed additive may contain about 0.3% to about 20% by weight of probiotic bacterial cells. Preferably the feed additive contains 7 % to 15 % by weight probiotic premix and most preferably about 10 % to 13 % by weight.

It will be further appreciated that the probiotic microorganisms of the present invention may not adhere to the intestinal epithelium. Thus in the absence of a repeat dosage, the bacteria remain in the gastrointestinal tract for maximal time of approximately 3-5 days and are considered to be a transient flora (see Figures 3a-d). The relatively rapid gastrointestinal-clearance time and inability to adhere to the gastrointestinal epithelium of the strains of the present invention, has the advantage of preventing the later development of bacteremia in, for example, immunocompromised

individuals. Fecal shedding assay as shown in Example 6 of the Examples section may be used to assess removal of the bacteria from the treated subject.

The bacterial strains and or compositions of the present invention can be included in a product identified for treating a particular disorder such as described above. Typically, the product is in the form of a package containing the bacterial cells or compositions including same, or in combination with packaging material. The packaging material is selected to retain bacterial viability and includes a label or instructions for, for example, use of the components of the package. The instructions indicate the contemplated use of the packaged component, as described herein for the methods or compositions of the invention, contents (e.g., genus, species, strain designation), minimum numbers of viable bacteria at end of shelf-life, proper storage conditions and corporate contact details for consumer information. The label may also provide information related to the freshness of the product. This information may include a date of manufacture, a "sell be" date or a "best before date". A "sell by" date specifies by which date the product should have been sold to the consumer. A "best before" date specifies by when the product should be disposed of by vendor or consumer. Alternatively or additionally "active labeling" may be used. For example, U.S. Pat. Nos. 4,292,916, 5,053,339 5,446,705 and 5,633,835 describe color changing devices for monitoring the shelf-life of perishable products. These devices are initiated by physically bringing into contact reactive layers so that the reaction will start, and this action can only conveniently be performed at the time of packaging. This approach is suitable for monitoring the degradation of foodstuffs which lose freshness throughout the entire distribution chain. U.S. Pat. No. 5,555,223 describes a process for attaching timing indicators to packaging, including the step of setting the timer clock at the exact time of production.

Depending upon the intended use, the product may optionally contain either combined or in separate packages one or more of the following components: colonization carriers, flavorings, carriers, and the like components. For example, the product can include the probiotic of the present invention for use in combination with a conventional liquid product, together with instructions for combining the probiotic with the formula for use in a therapeutic method.

The bacterial strains of the present invention can also be used as pharmaceutical delivery systems. It will be appreciated that such delivery systems are inherently safer than the use of attenuated pathogens in humans, including infants, the elderly and individuals whose immune function is impaired [Grangette (2001) Infect. Immun. 69:1547-1553].

The bacterial strains of the present invention can also be modified to express heterologous expression products using expression systems, which are well known in the art. This approach was used to reduce colitis in mice intragastrically administered with the IL-10-secreting *L. lactis* strain [Steidler (2000) Science 289:1352-1355].

As used herein the term "about" refers to ± 10 %.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998);

methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

BACKGROUND

The overall objective of this study is to develop an assay for detection of the M17 strain of *Escherichia coli* (M17) in fecal samples collected from mammalian sources. The approach used was to first isolate a spontaneously occurring nalidixic acid-resistant mutant derivative of M17 and second to develop a method for specific enumeration and confirmation of this strain from fecal samples.

Materials

Materials – Vendors of chemicals are listed in Table 4 below.

Table 4

Chemical	Source	Quality Item No.	CAS No.
Nalidixic Acid	Sigma-Aldrich	N-8878	
Agar	ACROS	400395000	9002-18-0
Agarose (PFGE)	BioWhittaker	50150	
Agarose (horizontal gels)	Fisher	BP160	
VRBA	Becton Dickson	211695	
Tris HCl (?)	Fisher	BP152	77-86-1
Boric Acid	Fisher	BP168	10043-35-3
EDTA	Fisher	BP120	6381-92-6
Sodium dodecyl-sulfate	EMD	7910	151-21-3
Phenol	Fisher	BP1750	108-95-2
Ethidium Bromide	Promega	H5041	
DNTPs[a]	Takara	4030	
PCR Buffer	Sigma	P-2192	

[a]deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP)

Vendor details of biological materials are listed in Table 5 below.

Table 5

Microbial Agent date	Source	Lot No	Production
M17	Benchmark Biolabs[a]	M17-01-001	04/07/2005
ECOR collection Thomas Whittam[b]			
AOS strains	John Maurer[c]		
ATCC202226 (DSM 12799)	American Type Culture Collection		

[a]Prairie Village, KS
[b]Dept. of Food Safety and Toxicology, Michigan State Univ
www.foodsafe.msu.edu/whittam/ecor/
[c]Dept. Of Avian Medicine, Univ. of Georgia College of Veterinary Medicine, Athens, GA.

Biological Reagents	Source	Catalog/Product No.
AFLP[a] Template Prep kit	Li-Cor[b],	B50623-01
Taq Polymerase	Takara[c]	R007A
Proteinase K	IBI[d]	5N0250
XbaI Restriction Enzyme	NEB[e]	R0145S
Lambda concatamers	NEB	N0350S
AFLP labeled primers	Li-Cor	
AFLP unlabeled primers	Li-Cor	

[a]Amplified Fragment Length Polymorphism
[b]Li-Cor, inc. 4308 Progressive Ave, Lincoln, NE, 68504
[c]Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan
[d]IBI/Shelton Scientific, 230 Long Hill Cross Rd., Shelton, CT 06484
[e]New England Biolabs, 240 Country Rd, Ipswich, MA 01938-2723

Disposable Materials – Vendor details of disposable materials are listed in Table 6 below.

Table 6

Item	Source	Item No.
Petri dishes	VWR Scientific	25384-302
Cryovials	Wheaton	985734
96-well PCR plates	GeneMate	T-3049-1
96-well Culture plates	Corning	3799
Micropipette tips 200 µL	DOT Scientific	ERY-0200
Micropipette tips 10 µL	Midwest Scientific	NKD-96-10
Microcentrifuge tubes	DOT Scientific	509-FTG
15 mL Sterile disp. Tubes	Fisher Scientific	14-959-70C

Equipment – Vendor details of laboratory equipment are listed infra.

Automated DNA Sequencers - Li-Cor/NEN model 4200 Global IR^2 automated DNA sequencer (item No. 9942-155). Dual laser/detector (685 nm and 785 nm diode lasers with silicon avalanche photodiode detectors), complete with Netwinder server (80 Gb storage, server software, operating system). Operating software: e-Seq Version (Item No. 9942-154).

Pulsed Field Gel Electrophoresis - Bio-Rad CHEF DRII Pulsed Field Gel Electrophoresis system + chiller system (item No. 170-3725). 100/120 V electrophoresis cell with drive module, control module, variable-speed pump, 14 x 13 cm casting stand with frame and platform, comb holder, 15-well, 1.5 mm thick comb, screened cap, disposable plug molds, and 12 ft Tygon tubing.

Horizontal Agarose Gel Electrophoresis - Owl Scientific, Model A1 Gator™ Large Gel Electrophoresis System

Gel Size: 13cm W x 25cm

Owl Scientific, Model B1A EasyCast™ Mini Gel Electrophoresis System Gel Size: 7cmW x 8 cm

Polymerase Chain Reaction Thermocyclers (Manufacturer, Location) - Whatman-Biometra T1, 96-well programmable thermocycler (item No 050-91).

Whatman-Biometra T Gradient, 96-well programmable temperature-gradient thermocycler (item No. 050-801)

Imaging system - Syngene Ingenious imaging system, 8 bit monochrome imaging camera with 768 x 582 pixel resolution, manual zoom lens, darkroom + 20 X

30 cm UV2 (302 nm/365 nm wavelength) transilluminator, GeneSnap image capture software and GeneTools image analysis software.

EXAMPLE 1

Isolation of a nalidixic acid-resistant mutant of the M17 Escherichia coli strain

Experimental Procedures

The M17 strain of *Escherichia coli* is known to be sensitive to the antibiotic nalidixic acid. Only a small number of *E. coli* strains isolated from human clinical samples are known to be resistant to nalidixic acid and other quinilone antibiotics (4, 8, 9). Because nalidixic acid resistance, which is associated with mutations in gyrA or parC, occurs spontaneously *in vitro* and is only observed at low frequencies in clinical samples, it is well suited as a simple means for marking the M17 strain.

To isolate a nalidixic acid-resistant mutant derivative of M17, an M17 *E. coli* culture was grown for 16 hours at 37 °C in Luria Broth and 0.1 mL portions of the culture were then spread onto the surface of Luria agar supplemented with 15 μg/mL Nalidixic acid. The agar plates were then incubated for 16 hours at 37 °C. Two colonies (M17 15-1 and M17 15-2) were then selected, streaked onto Luria agar supplemented with 15 μg/mL nalidixic acid, and inoculated into 5 mL of Luria broth with 15 μg/mL of nalidixic acid and incubated for 16 hours at 37 °C. 0.1 mL portions of the Luria broth cultures were then spread onto the surface of Luria agar plates supplemented with 50 μg/mL nalidixic acid. A total of four colonies were chosen from each original parent (M17 15-1 or M17 15-2), these colonies were labeled M17 50-1 thru M17 50-4 (from M17 15-1 parent) and M17 50-5 thru M17 50-8 (from the M17 15-2 parent). The M17 50-1 thru M1750-8 colonies were then streaked onto Luria agar with 50 μg/mL nalidixic acid, inoculated into Luria broth supplemented with 50 μg/mL nalidixic acid, and incubated for 16 hours at 37 °C. Portions of each Luria broth culture (0.1 mL) were then spread onto the surface of Luria agar plates supplemented with 100 μg/mL nalidixic acid. The plates were incubated overnight at 37 °C. A single colony from the Luria agar with 100 μg/mL nalidixic acid plates derived from each parental strain (M17 50-1 thru M17 50-8) was chosen, and labeled

M17 100-1 thru M17 100-8, streaked onto Luria agar supplemented with 100 μg/mL nalidixic acid and grown for 16 hours at 37°C.

Preparation of freezer stocks of nalidixic acid-resistant M17 derivatives. Single colonies from the M17 15-1, M17 15-2, M17 50-1 thru M1750-8 and M17 100-1 thru M17 100-8 cultures were grown in Luria broth supplemented with the appropriate amount of naliixic acid and (15, 50, or 100 μg/mL) and grown to an optical density of 0.5 (600 nm). From each culture, 0.7mL was removed to sterile cryogenic tubes, mixed with 0.3mL sterile 50% glycerol, and rocked at room temperature for 45 minutes. The glycerol-treated cells were then stored at -80°C.

Results

From the parental nalidixic acid-sensitive M17 strain, two individual colonies resistant to 15 μg/mL of nalidixic acid were chosen. A series of four derivatives each from the M15 15-1 and M17 15-2 parental strains were then isolated which were resistant to 50 μg/mL nalidixic acid. A single derivative, resistant to 100 μg/mL of nalidixic acid was then obtained from each of the M17 50-1 thru M17 50-8 strains. A 1 mL aliquot of each M17 nalidixic acid-resistant derivative listed in Table 7 below was then frozen at -80 °C for long-term storage.

Table 7 - Nalidixic acid resistant derivatives of M17

Stock Strain	Nalidixic acid concentration in isolation plate	Parent
M17 15-1	15 μg/mL	M17
M17 15-2	15 μg/mL	M17
M17 50-1	50 μg/mL	M17 15-1
M17 50-2	50 μg/mL	M17 15-1
M15 50-3	50 μg/mL	M17 15-1
M17 50-4	50 μg/mL	M17 15-1
M17 50-5	50 μg/mL	M17 15-2
M17 50-6	50 μg/mL	M17 15-2
M17 50-7	50 μg/mL	M17 15-2
M17 50-8	50 μg/mL	M17 15-2
M17 100-1	100 μg/mL	M17 50-1
M17 100-2	100 μg/mL	M17 50-2
M17 100-3	100 μg/mL	M17 50-3
M17 100-4	100 μg/mL	M17 50-4
M17 100-5	100 μg/mL	M17 50-5
M17 100-6	100 μg/mL	M17 50-6
M17 100-7	100 μg/mL	M17 50-7
M17 100-8	100 μg/mL	M17 50-8

EXAMPLE 2

Characterization of the M17$_{SNAR}$ strains of the present invention

A series of molecular genetics and biochemical assays were run to characterize the strains of the present invention.

Experimental Procedures

Pulsed Field Gel Electrophoresis (PFGE) Confirmation of Nalidixic acid-resistant M17 derivatives - PFGE was performed to confirm nalidixic acid-resistant derivatives of M17 are indeed M17 derivatives. PFGE was performed using the CDC protocol, "Laboratory Protocol for Molecular Subtyping of Escherichia coli O157:H7 by Pulsed Field Gel Electrophoresis." see www.cdc.gov/pulsenet/protocols.htm Centers for Disease Control and Prevention. Standardized molecular subtyping of foodborne bacterial pathogens by pulsed-field gel electrophoresis: a manual. Atlanta: National Center for Infectious Diseases; 1996 (updated 2000). Briefly, a Luria agar plate was streaked from frozen stock cultures of the M17 parent strain and the M17 100-1 thru M17 100-8 nalidixic acid resistant derivatives onto Luria agar or Luria agar supplemented with 10 μg/mL Nalidixic acid and grown for 16 hours at 37 °C. Using a sterile microbiological loop, about 20 μL of cells were transferred from each plate to a tube containing 1 mL of Suspension Buffer (100mM Tris, 100mM EDTA, pH 8.0). The suspension was adjusted to an absorbance value at 610 nm of 1.35 using additional cells or suspension buffer as necessary. The cell suspension (0.4 mL) from each strain was then mixed with 20 μL of Proteinase K (stock concentration 20 mg/mL), and inverted several times. 0.4 mL of molten agarose was then mixed with the cells and immediately dispensed into the plug molds of the CHEF II-DR PFGE apparatus. After solidification, the plugs were then removed into 50 mL screw cap tubes using a spatula and 5 mL of Cell Lysis Buffer [50mM tris, 50mM EDTA, 1% Sarkosyl (sodium lauryl sarcosine) pH 8.0]. Twenty-five microliters (25 μl) of Proteinase K (20 mg/mL stock concentration) was then added and the plugs were incubated for 2 hours at 54 °C. The lysis buffer was removed by decanting and the plugs were washed briefly by swirling in 10 mL of sterile water. The water was then decanted and the plugs were washed 4 times for 15 minutes each in 10 mL of TE

buffer. After a final rinse in 10 mM Tris, 1 mM EDTA pH 8.0 (TE), the plugs were stored at 4 °C in sterile TE buffer.

Restriction digests were performed by placing 2 mm slices of the plug into a sterile 1.5 mL microcentrifuge tube and adding 0.1 mL of Restriction Buffer. Restriction Buffer for XbaI restriction enzyme included 10mM Tris, 10mM MgCl2, 50mM NaCl, 1mM Dithiothreitol, pH 7.9. After 15 minutes of incubation at 37 °C, the Restriction Buffer was decanted and 0.1 mL of Restriction Buffer + 30 units of XbaI restriction enzyme was added. The samples were then incubated for 2 hours at 37 °C. After incubation, samples were decanted and 0.2mL of 0.5X TBE buffer was added.

The analytical agarose gel was cast by mixing a slurry of 1% SKG agarose in 0.5X TBE and melting the agarose. After cooling in a 60 °C water bath, the agarose was then poured into the CHEF II-DR gel form and the comb carefully placed into the molten gel. After 1 hour of solidification at room temperature, the comb was removed and the restriction-digested plugs placed into the appropriate wells, being sure to push the gel slice to the front of the well and removing any bubbles. A small volume of molten agarose was then added to fill the remaining area in the wells. The gel was then placed into the CHEF DRII PFGE tank, being sure to place the gel within the gel frame of the chamber. The chamber was then filled with 0.5 X TBE (1 M Tris, 1 M Boric Acid, 20 mM EDTA, pH 8.3 diluted to 0.5X concentration with water for electrophoresis).and electrophoresed under the following conditions:

Initial A time: 2.2s
Final A time: 54.2s
Start Ratio: 1.0
Voltage: 200V (6V/Cm)
Run Time: 22 hr

Following electrophoresis, the gel was stained for 15 minutes in a solution of 0.02 μg/ml Ethidium bromide and destained for 30 minutes in water. The stained DNA was visualized by placing the gel onto a 302 nm UV lightbox and imaged with CCD camera.

Amplified Fragment Length Polymorphism (AFLP) - AFLP reactions were performed according to Li-Cor, Inc. Document #988-07304, Rev. 1. Template DNA

was prepared by standard methods and redissolved in 10 mM Tris, 1 mM EDTA pH 7.5. Genomic DNA was extracted from the bacterial strains by standard methods (5). The DNA samples were dissolved in 10 mM Tris-0.1 mM EDTA pH 8.0. A total of 100 ng of DNA from each sample was digested with EcoRI and MseI. Double-stranded, synthetic DNA adapters, containing short single strand sequences complementary to the EcoRI and MseI overhangs were then ligated to the digested DNA fragments. Fragments with ligated adapters were then diluted and amplified by PCR in pre-amplification reactions using PCR primers specific for the adapters. The amplified fragments then served as templates for selective amplification in which fluorescently labeled primers are used in conjunction with unlabeled primers. The selective amplification uses primers that are complementary to the adapter sequences with an additional 2 bases at the 3'end. This selectively amplified (and labels) DNA fragments in which the 2 bases immediately adjacent to the original EcoRI or MseI site are complementary to the 3' base of the primers. Lastly, the labeled selective amplification products were resolved by denaturing polyacrylamide gel electrophoresis on a Li-Cor/NEN 4200 global analyzer (automated DNA sequencer).

Results

To confirm the genetic relationship of the M17 100-1 thru M17 100-8 strains to the original M17 parental strain, PFGE analysis was performed on each strain and the parental M17 strain. As shown in Figure, The M17 100-1 thru M17100-8 derivatives are all indistinguishable from the M17 parental strain.

Based on PFGE Confirmation, all nalidixic acid-resistant derivatives are genetically indistinguishable from the M17 parent. The M17 parent and M17 100-1 thru M17 100-8 derivative strains were then streaked onto Violet Red Bile Agar to test for lactose fermentation. All strains were lactose positive. Therefore, the M17 100-8 strain was chosen as the nalidixic acid-resistant M17 derivative for all further studies. This strain was designated $M17_{SNAR}$ (Sucrose-positive, Nalidixic Acid Resistant).

Further analysis of the genetic relatedness of M17 and the $M17_{SNAR}$ derivative was conducted by AFLP analysis. Total DNA from the M17 parental, $M17_{SNAR}$, 14 different ECOR strains (6) and two genetically unrelated serotype O2 strains (AOS1

and AOS19) was subjected to AFLP using the fluorescently labeled EcoRI-A and unlabeled MscI-GA primers. The reaction products were resolved by gel electrophoresis and the resulting image used to compare the banding patterns. As shown in Figure 2, all of the resolvable, fluorescently-labeled AFLP products from M17 and $M17_{SNAR}$ are identical in size, whereas the fragment patterns produced from the different ECOR strains and the AOS strains show several differences compared to M17 and $M17_{SNAR}$. Therefore, the M17 parent and $M17_{SNAR}$ derivative are indistinguishable by both PFGE and AFLP.

EXAMPLE 3

DNA sequence analysis to identify unique genetic signatures of the $M17_{SNAR}$ genome

Experimental Procedures

Preparation of Genomic DNA for DNA sequence analysis - For DNA sequencing, total genomic DNA from the M17100-8 SNAR isolate was prepared. This clone was grown overnight on Luria agar with 100 μg/mL nalidixic acid. Following 16 hours of growth at 37 °C, a loopful of cells was transferred to 500 mL of Luria broth supplemented with 100 μg/mL nalidixic acid and grown for 18 hours at 37°C with shaking at 200 rpm. The cells were then harvested by centrifugation in a Backman J2 high speed centrifuge at 6,000 rpm for 10 minutes using 250 mL bottles in a JA10 rotor. The cells were then resuspended in 25 mL of 10mM Tris-1mM EDTA (pH 7.5) with 2 mg/mL lysozyme. After 10 minutes of incubation at room temperature, 2.5 mL of 1% Sodium Dodecyl-Sulfate supplemented with 5 mg/mL Proteinase K and incubated at 50°C for 90 minutes. 25 mL of phenol (saturated with 10mM tris-1mM EDTA pH 8.0) was then added and the bottles were rocked for 3 hours on high speed. The phases were then separated by centrifugation at 8,000 rpm for 15 minutes in a Beckman J2 High-speed centrifuge using a JA10 rotor. The aqueous phase (20 mL) was then removed to a beaker and 2 mL of 3 M Sodium Acetate (pH 5.2) was added and mixed with swirling. The DNA was then precipitated by the addition of 40 mL of ethanol. Precipitated DNA was spooled onto a glass from the interface and the spooled DNA washed by submerging several times into 70% ethanol. The DNA was finally dissolved in 1 mL of 10mM Tris-1mM EDTA.

Dissolved DNA was then dialyzed for 2 hours against sterile water (1Liter) for three consecutive changes of water. DNA was then transferred to the laboratory of Dr. Vivek Kapur - The Biomedical Genomics Center at the University of Minnesota sequence analysis.

Identification of M17$_{SNAR}$ unique sequences from the genome sequence - A single file containing all of the assembled DNA sequences (contigs) from alignments of shotgun reads from the M17$_{SNAR}$ libraries was obtained from Dr. Vivek Kapur (A. Benson computer, D:/Midland Genomics/BioBalance/M17 Genome data\0812_0003.contigs.fasta.txt). Individual contigs resulting from alignment of >200 sequence runs were obtained from the file and used to create text files in fastA format. The fastA files were then BLAST searched against the *E. coli* CFT073 genome sequence (GenBankAccession No. NC004431) using the BLAST2 algorithm (7) available at the NCBI website (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Coordinates from the alignments were then entered into Microsoft Excel spreadsheets and segments of non-alignment were determined by identifying coordinates of sequence segments that do NOT produce BLAST alignments. Only segments of non-alignment > 1,000 bases were considered for further analyses.

Segments from each M17$_{SNAR}$ contig corresponding to non-aligning regions were obtained from the contig files using the Extract DNA program available from the Pathogenomics Sequence Analysis facility of the University of Minnesota (www.pathogenomics.ahc.umn.edu/ExtractDna.htm). Each of the non-aligning segments was then used to create a .tExt file in fastA format and finally used to search the entire Genbank database at NCBI using the BLASTn algorithm (1, 2). Segments not yielding significant alignment were then considered as candidates for regions of genome sequence unique to M17$_{SNAR}$.

Design of PCR primers for amplification of M17SNAR unique genome regions - From the text files containing the unique segments of M17$_{SNAR}$, PCR primers were designed using the PRIME program from the Wisconsin Package (Genetics Computer Group package www.accelrys.com/products/gcg/). The parameters used in designing the primers are as follows:

PCR primers should produce products between 500 bases and 3 kilobases in length.

PCR primers should be positioned within the region of unique sequence.

PCR primer pairs should have melting points that are within 2 °C of each other.

PCR primers from each unique region should give products that are of distinct size so they can be multiplexed. Potential primer combinations are listed in Table 11 below.

Optimization and validation of M17SNAR unique genome regions by PCR

In order to optimize the PCR reactions for detection of $M17_{SNAR}$-specific genome segments (M17SSGS), a single PCR primer combination from the list of candidates (see Table 11 below) was tested against $M17_{SNAR}$ chromosomal DNA to determine if a specific product of the predicted size was produced. The reactions were run at different melting temperatures using the TGradient thermocycler (Biometra). The gradients were centered at 59 °C with 15 °C variance on the low and high ends. The reactions were run using 1 ng of $M17_{SNAR}$ DNA in a 20 µL reaction volume with 1 X PCR buffer (containing 2.5 mM $MgCl_2$ final concentration), 250 µM dNTPs, 1 unit of Taq DNA polymerase, and 1 µM each primer. The reaction volume was made to 20 µL for each reaction using sterile water. Reactions were heated to 95 °C for 2.5 minutes and then 30 cycles of 95 °C for 30 seconds, melting temperature (56 or 63 degrees depending on primer set) for 45 seconds, 45 seconds at 72 °C. After 30 cycles, the reaction was extended for 5 minutes at 72 °C and held at 4 °C until ready for gel electrophoresis.

Once terminated PCR reactions were supplemented with 2 µL of loading dye (0.21 % Bromphenol Blue, 0.21 % Xylene cyanol, 50% glycerol). A total of 15 µL of the reactions was then loaded onto a 0.8 % agarose gel prepared in 1 X TAE containing 1 ng/mL of ethidium bromide. The gel was then electrophoresed for 1.5 hours at 100 Volts/Cm. The electrophoresed PCR products were then visulalized by placing the gel onto a 302 nm UV lightbox and imaged with CCD camera.

Results

Although the fragment patterns derived from different AFLP primer combinations can distinguish $M17_{SNAR}$ from different *E. coli* strains, a more simplistic approach to distinguish $M17_{SNAR}$ from other *E. coli* strains is to use a combination of

the nalidixic acid resistance (where the trait of nalidixic acid resistance is used to selectively grow resistant bacteria—i.e. identify only those bacteria in the feces which can grow in the presence of nalidixic acid—and then confirm their identity as $M17_{SNAR}$ using specific genetic tests) and to selectively grow nalidixic acid-resistant bacteria from fecal samples and confirm their identity as $M17_{SNAR}$ using a genetic test for a DNA segment that is unique to the M17 parent and $M17_{SNAR}$ derivative. To identify a segment of DNA unique to M17 and $M17_{SNAR}$, the $M17_{SNAR}$ genome was subjected to whole genome DNA sequence analysis. Genomic DNA was extracted from $M17_{SNAR}$. The DNA was subsequently physically sheared into different size fragment lengths and three different clone libraries were generated, each having different average insert sizes (4 Kilobases, 10 Kilobases, and 40 Kilobases). Each of these libraries was then subjected to high-throughput shotgun DNA sequence analysis and the sequence reads were assembled into large contiguous DNA sequences based on their overlap. As is common practice, the shotgun DNA sequencing phase included DNA sequence analysis of enough clones such that each segment of the entire genome would be sequenced multiple times in independent overlapping clones.

As illustrated in Table 8 below [Ewing, B., L. Hillier, M. Wendl, and P. Green. 1998. Base-calling of automated sequencer traces using Phred. I. Accuracy assessment. Genome Res Vol. 8 (3), 175-185], the high-throughput shotgun sequencing phase of the *E.coli* $M17_{SNAR}$ genome resulted in 57,408 distinct high-quality 'reads' representing 36,265,538 bases with good quality scores, providing an approximately 8-fold coverage of the genome. Based on the total number of Phred20 bases, the paired-end sequence reads from the 4 Kb genome library provided the most coverage of the genome sequence. The 10kb and Fosmid libraries provided larger physical links from paired-end 'reads' and facilitated ordering and orienting contiguous sequence 'blocks' in the assembly process.

Table 8 - DNA Sequence 'reads' generated by the high-throughput sequencing of the three libraries

	Libraries		
	4kb insert	10kb insert	Fosmid (40kb insert)
Total 'reads'	27,648	13,728	16,032
Pass rate*	91.54%	83.75%	85.03%
Average Phred20 bases** per 'read'	725	763	670
Total Phred20 bases	18,349,009	8,772,363	9,144,166

* Pass rate: defined as a 'read' containing >100 cumulative Phred20 bases**

** Phred20 bases: Bases which receive a quality score of 20 or more when subjected to Phred analysis, a base-calling program developed at the University of Washington Genome Center.

During the assembly phase, sequence data, vector sequences were removed and the sequence was quality screened based on Phred quality score information [see Ewing B, Green P: Basecalling of automated sequencer traces using phred. II. Error probabilities. Genome Research 8:186-194 (1998); Ewing B, Hillier L, Wendl M, Green P: Basecalling of automated sequencer traces using phred. I. Accuracy assessment. Genome Research 8:175-185 (1998)].

All the sequence 'reads' from the libraries were first compared to each other. Identities between the sequences of different 'reads' were noted, and then used to align the sequences into contiguous stretches of sequence called contigs. Because of small variations in the quality of the sequence from independent reads, two different 'reads' of the same segment of DNA may not be identical. Therefore, enough clones from the different libraries are sequenced to generate multiple overlapping reads of each base so that it is independently confirmed.

Contig building software, which builds contigs based on the "quality" of each base in a 'read', was used to ultimately build the DNA sequence into large contiguous stretches of sequence. Any gaps, discrepancies or ambiguities in the sequence were also identified. Contigs were then ordered and linked together into larger supercontigs by using paired 'reads' lying in different contigs. Using this approach, a total of 464 contigs were assembled and the details are listed in Table 9 below. Whole genome assembly was performed using the Paracel Genome Assembler™, version 2.6.2, coupled with the Agencourt's LIMS system while, Paracel's scaffold viewer and Consed (version 13.0) were used to finish the assembly.

Table 9. Summary of the sequence assembly

Contigs	Total #	Total length
Supercontigs	71	4420860
Contigs (≥2 kb)	313	4706396
Contigs (<2 kb)	80	na*
Total # of contigs	464	Na

* na, Not applicable

Strategy to identify unique sequences in the M17$_{SNAR}$ strain – Parallel approaches were used to identify potential unique sequences.

All 464 total contigs were provided electronically as **.txt* files of the individual contig sequences in FASTA format (www.ncbi.nlm.nih.gov/blast/html/search.html). A pairwise BLAST analysis was consequently done of entire contigs against the *E. coli* CFT073 genome sequence and subsequently identified the non-aligning regions from the M17 contigs. These non-aligned sequences were then used in pairwise BLAST to identify any sequence homology against the *E. coli* MG1655 (K-12) and *E. coli* EDL933 (O157:H7) and Sakai (O157:H7) genome sequences. Segments not showing significant alignment (sequence homology) to either the *E. coli* CFT073, K-12, or O157:H7 genomes were then used in a BLAST search against the entire nr NCBI database (www.ncbi.nlm.nih.gov/BLAST/).

In a parallel approach, a BLAST analysis was effected with each of the 464 contigs first split up into 200 bp (base pair) fragments and then individually blasted against two specialized databases. The first database constructed was an '*E.coli*' database, in which four strains of *E.coli* (www.ncbi.nlm.nih.gov/genomes/lproks.cgi) were included to create a database named NCBIrefseq_ecoli.dna *(Escherichia coli* strains included were CFT073, K12, O157:H7 Sakai and O157:H7 EDL933). For the second database, all bacterial genomes present in the NCBI database were consolidated to create a 'Bacterial' database and named NCBIrefseq_bacteria.dna. Next, each of the 200 bp fragments was subjected to a blastn analysis against both consolidated NCBI databases of both *E. coli* and bacteria, described above. Sequences with 'no hits,' were identified to be unique sequences.

Results of alignments: identification of M17$_{SNAR}$ unique genome segments - Using the large-fragment, pair-wise alignment approach several segments from different contigs of the M17$_{SNAR}$ genome sequence were identified which did not yield significant alignments to any sequence from all publicly available sequences in

the nr database of NCBI. These candidate unique segments are indicated in Table 10, below. These segments were then referred to as candidate $M17_{SNAR}$ unique sequences.

Table 10 Coordinates of aligning and non-aligning unique DNA sequences from the M17 genome sequence

Contig Number	Contig Length	Coordinates of Unique segments	Unique segment length
10	315,599	292,869-294,947	2,078
11	128,617	45,040-46,133	1,093
17	67,917	3,603-14,778	11,175
17	67,917	34,624-54,505	19,881
17	67,917	65,633-67550	1,917
36	130,495	88,527-93,269	4,743
41	75,562	23,625-25,532	1,907
41	75,562	30,174-36,945	6,771
127	40,938	21,584-24,327	2,743
291	32,242	3,566-5,534	1,968
291	32,242	19,653-21,193	1,540
291	32,242	27,379-30,296	2,917
291	32,242	30,418-31598	1,180

Results from optimization and validation of PCR analysis for $M17_{SNAR}$ unique segments - To experimentally test the uniqueness of the test the uniqueness of the candidate $M17_{SNAR}$ unique sequences, PCR primers were designed to amplify segments within several of the unique regions. The PCR primers were then used in PCR reactions performed on the M17 and $M17_{SNAR}$ strains to first optimize the PCR reactions. Optimizations were performed using temperature gradients centered at 59 °C and +/- 7.5 °C above and below this temperature. The PCR products from each reaction were run alongside one another on an analytical agarose gel, stained with ethidium bromide, and imaged over a 302 nm UV lightbox using a CCD camera. The relative intensity of fluorescence from the stained DNA bands was quantified using GeneTools software (Syngene) software. As shown in Table 11, below, optimal PCR conditions were developed for PCR primers detecting unique regions in Contig 10, Contig 11, Contig 13, Contig 36, Contig 41, Contig 127, and Contig 291. The Contig 127 PCR primers, which have the highest average melting temperature and produced optimal PCR reactions at the upper end of the optimization curve, were subsequently chosen for further validation against E. coli strains representing the genetic diversity of naturally occurring E. coli populations.

Table 11 - Optimization of PCR assays for candidate M17SNAR unique regions

Primer combination (SEQ ID NO.)	Contig_coordinates[a]	PCR product length	Predicted melting temperature[a]	Optimized PCR melting temperature[b]
C10FOR GAAAAACCCACCGACATCAAC (10) C10REV ATATCAGCACCCCCACCAAG(11)	C10_292,453 C10_294,908	2,455	65.2 65.8	63
C11FOR TAACCAGCGGCATCATCAG(12) C11REV CGGCAAGAAAAACGAATCAC(13)	C11_44,112 C11_46,326	2,214	64.8 64.3	63
C13FOR TCCAGCAAGAAAACAACCAC(14) C13REV GATACTGATGATGCCACCAC(15)	C13_38,896 C13_41,915	3,019	62.7 60.4	63
C36FOR CATCCACAATTCCCCAATCC(16) C36REV ACCTCTGCTGAACACATAAAC(17)	C36_89,356 C36_90,461	1,105	66.1 58.3	53
C41FOR CAAGCAGGGAAGCATCAAC(18) C41REV TATCAACAGGAGCCACCAC(19)	C41_32,457 C41_33,993	1,536	63.4 61.2	63
C127FOR TACCCCTCATTGCTCATCCC(20) C127REV GATTACCCCACAAAAACTGACC(21)	C127_22,666 C127_23,821	1,155	66.0 63.1	63
C291FOR TGAGCAGTGCCATCAACAG(22) C291REV CAAAAGCCGAATTAAACGGAG(23)	C291_28,506 C291_29,972	1,466	63.9 64.3	63

[a]Coordinate for the 5' nucleotide of the respective primer is indicated
[b]melting temperature predicted from the PRIME program in the GCG package
[b]melting temperature producing most significant amount of PCR product

EXAMPLE 4

The M17SNAR strains of the present invention are molecularly distinct from other E. coli strains

Experimental Procedures

Once the reactions were optimized for each PCR primer combination, the combinations were confirmed by testing them in PCR reactions against a panel of E. coli strains designed to represent the genetic diversity of the total E. coli population (ECOR collection) and to represent common serotype O2 strains. The collection consists of the 72 ECOR strains, described originally by Ochman and Selander in 1984 (6), which has been extensively studied and is generally regarded as representative of the population structure of the species. A set of five different strains having an O2 serotype was also used to test for the uniqueness of the $M17_{SNAR}$ segment in genetically unrelated E. coli strains that share the O2 serotype with $M17_{SNAR}$. Each strain of the 77-strain set was grown for 16 hours at 37 °C in Luria

Broth and the cells harvested by centrifugation in a JA10 rotor. DNA was then extracted from each strain according to standard methods (5). DNA samples were dissolved in 10 mM Tris-1 mM EDTA pH 8.0 and stored at 4 °C. For each PCR reaction, the DNA samples were diluted 1:100 in sterile water and a 2 µL volume of the diluted DNA was added to the reaction. The reaction mixtures each contained 2 µL of the diluted DNA, 1 µL each of the relevant primers (final concentration 1uM) 2 µL of 10X PCR buffer (Takara), 2 µL of 250mM dNTP mixture (Takara), 1 unit of Taq DNA polymerase (Takara) and 11 µL of sterile water. The reactions were then cycled in a Biometra thermocycler using the following cycling conditions: 2.5 minutes at 95 °C, followed by 30 cycles of 30 seconds at 95 °C, 45 seconds at 53 °C (Primer combination C127) or 56 °C (Primer combination C291), 45 seconds at 72 °C. A final extension of 5 minutes at 72 °C was performed after the thirty cycles and the reactions were held at 4°C until ready for gel electrophoresis.

To the completed PCR reactions, 2 µL of loading dye (0.21 % Bromphenol Blue, 0.21 % Xylene cyanol, 50 % glycerol) was added. A total of 15 µL of the reactions was then loaded onto a 0.8 % agarose gel prepared in 1 x TAE containing 1 ng/mL of ethidium bromide. The gel was then electrophoresed for 1.5 hours at 100 Volts/Cm. The electrophoresed PCR products were then visulalized by placing the gel onto a 302 nm UV lightbox and imaged with CCD camera.

To experimentally test candidate $M17_{SNAR}$ unique sequences, PCR primers were designed to amplify segments within several of the unique regions. The PCR primers were then used in PCR reactions performed on the M17 and $M17_{SNAR}$ strains to first optimize the PCR reactions. Optimizations were performed using temperature gradients centered at 59°C and +/- 7.5°C above and below this temperature. The PCR products from each reaction were run alongside one another on an analytical agarose gel, stained with ethidium bromide, and imaged over a 302 nm UV lightbox using a CCD camera. The relative intensity of fluorescence from the stained DNA bands was quantified using GeneTools software (Syngene) software. As shown in Table 5, optimal PCR conditions were developed for PCR primers detecting unique regions in Contig 10, Contig 11, Contig 13, Contig 36, Contig 41, Contig 127, and Contig 291. The Contig 127 PCR primers, which have the highest average melting temperature and produced optimal PCR reactions at the upper end of the optimization curve, were

subsequently chosen for further validation against *E. coli* strains representing the genetic diversity of naturally occurring *E. coli* populations.

To validate the specificity of the Contig 127 PCR assay, the optimized Contig 127 PCR reactions were next tested against genomic DNA from a panel of *E. coli* strains representing the genetic diversity of naturally occurring *E. coli* populations (ECOR collection) as well as additional serotype O2 strains (AOS strains) that are genetically unrelated to the serotype O2 $M17_{SNAR}$ strains. Purified genomic DNA from each of the 72 ECOR strains, each AOS strain, the $M17_{SNAR}$ strain, and the M17 parental strain were subjected to PCR in individual reactions with the Contig 127 primers using the following conditions:

Template DNA	100 ng
C127FOR	1pmol/uL
C127REV	1pmol/uL
1 X PCR buffer	
dNTPs	250uM
Taq polymerase	1 Unit
Water	to 20 µL

The PCR reactions were heated for 2.5 minutes at 95°C followed by 30 cycles of 95°C for 30 seconds, 63°C for 45 seconds, 72°C for 45 seconds. The reactions were extended for 5 minutes at 72°C and finally held at 4°C until electrophoretic separation. For agarose gel electrophoresis, loading dye was added and the reactions were loaded into a 0.8% agarose gel. After electrophoresis, the gels imaged over a UV lightbox using CCD camera. As shown in Table 12, only M17 and $M17_{SNAR}$ produced the expected 1,155 base PCR product from the Contig 127 PCR assay. All other strains failed to yield any PCR product from this reaction. Based on the strains that were tested in this validation, our results indicate that the Contig 127 PCR assay is highly selective for $M17_{SNAR}$.

Results

Table 12 below, summarizes the results of contig 127 analysis in the strains of the present invention and other *E. coli* strains tested.

Table 12 - Validation of Contig_127 PCR reaction on M17$_{SNAR}$, ECOR strains, and additional serotype O2 strains.

Isolate	O	H	Host	Contig_127 PCR result[a]
M17	2	HN	Human	+
M17SNAR	2	HN	Human	+
AOS1	2	HN	Avain	-
AOS11	2	HN	Avain	-
AOS19	2	HN	Avian	-
AOS29	2	HN	Avain	-
AOS36	2	HN	Avain	-
ECOR-01	ON	HN	human (Female, 19yr)	-
ECOR-02	ON	H32	human (Male)	-
ECOR-03	O1	NM	dog	-
ECOR-04	ON	HN	human (Female, 5yr)	-
ECOR-05	O79	NM	human (Female, 56yr)	-
ECOR-06	ON	NM	human (Male, 8yr)	-
ECOR-07	O85	HN	orangutan	-
ECOR-08	O86	NM	human (Female, 20yr)	-
ECOR-09	ON	NM	human (Female)	-
ECOR-10	O6	H10	human (Female)	-
ECOR-11	O6	H10	human (Female)	-
ECOR-12	O7	H32	human (Female)	-
ECOR-13	ON	HN	human (Female)	-
ECOR-14	OM	HN	human (Female)	-
ECOR-15	O25	NM	human (Female)	-
ECOR-16	ON	H10	leopard	-
ECOR-17	O106	NM	pig	-
ECOR-18	O5	NM	Celebese ape	-
ECOR-19	O5	HN	Celebese ape	-
ECOR-20	O89	HN	steer	-
ECOR-21	O121	HN	steer	-
ECOR-22	ON	HN	steer	-
ECOR-23	O86	H43	elephant	-
ECOR-24	O15	NM	human (Female)	-
ECOR-25	ON	HN	dog	-
ECOR-26	O104	H21	infant	-
ECOR-27	O104	NM	giraffe	-
ECOR-28	O104	NM	human (Female, 4yr)	-
ECOR-29	O150	H21	kangaroo rat	-
ECOR-30	O113	H21	bison	-
ECOR-31	O79	H43	leopard	-
ECOR-32	O7	H21	giraffe	-
ECOR-33	O7	H21	sheep	-
ECOR-34	O88	NM	dog	-
ECOR-35	O1	NM	human (Female, 36yr)	-
ECOR-36	O79	H25	human (Female, 20yr)	-
ECOR-37	ON	HN	marmoset	-
ECOR-38	O7	NM	human (Female, 21yr)	-
ECOR-39	O7	NM	human	-
ECOR-40	O7	NM	human	-
ECOR-41	O7	NM	human (Female, 22yr)	-
ECOR-42	ON	H26	human (Male)	-
ECOR-43	ON	HN	human (Female)	-
ECOR-44	ON	HN	cougar	-
ECOR-45	ON	HM	pig	-

Isolate	O	H	Host	Contig_127 PCR result[a]
ECOR-46	O1	H6	ape	-
ECOR-47	OM	H18	sheep	-
ECOR-48	ON	HM	human (Female)	-
ECOR-49	O2	NM	human (Female)	-
ECOR-50	O2	HN	human (Female)	-
ECOR-51	O25	HN	infant	-
ECOR-52	O25	H1	orangutan	-
ECOR-53	O4	HN	human (Female, 4yr)	-
ECOR-54	O25	H1	human	-
ECOR-55	O25	H1	human (Female)	-
ECOR-56	O6	H1	human (Female)	-
ECOR-57	ON	NM	gorilla	-
ECOR-58	O112	H8	lion	-
ECOR-59	O4	H40	human (Male)	-
ECOR-60	O4	HN	human (Female)	-
ECOR-61	O2	NM	human (Female)	-
ECOR-62	O2	NM	human (Female)	-
ECOR-63	ON	NM	human (Female)	-
ECOR-64	O75	NM	human (Female)	-
ECOR-65	ON	H10	Celebese ape	-
ECOR-66	O4	H40	Celebese ape	-
ECOR-67	O4	H43	goat	-
ECOR-68	ON	NM	giraffe	-
ECOR-69	ON	NM	Celebese ape	-
ECOR-70	O78	NM	gorilla	-
ECOR-71	O78	NM	human	-
ECOR-72	O144	H8	human	-

[a]+ 1,155 base PCR product present, - No PCR product

EXAMPLE 5

Enumeration and confirmation of $M17_{SNAR}$ in spiked fecal samples Experimental Procedures

Experimental Procedures

A combination of selective plating of on Violet Red Bile Agar (VRBA) and PCR confirmation was used to determine if $M17_{SNAR}$ could be selectively enumerated in a spiked fecal sample. For these experiments, a composite fecal sample was prepared by mixing 10 gram samples from 100 independent human fecal samples into a single composite. The composite was mixed for three 1-minute pulses in a Waring blender and the resulting slurry was distributed in approximately 25 mL aliquots into sterile 50 mL conical tubes. Herein, these samples are referred to as the fecal composite sample. The aliquots of composite sample were stored at -80 °C.

For testing, aliquots of fecal samples were thawed and diluted in triplicate by serial 10-fold dilutions into sterile 0.1 % peptone. 0.1 mL samples of each dilution were then plated in duplicate onto the surface of Luria agar, Luria agar + 75 μg/mL nalidixic acid, VRBA, VRBA + 25 μg/mL nalidixic acid. $M17_{SNAR}$ cells were spiked into the 10-1 dilution of two independent fecal composite aliquots. For the spiking experiment, a suspension of $M17_{SNAR}$ cells was prepared by scraping a single colony of $M17_{SNAR}$ cells from a culture that had been streaked onto Luria agar + 75 μg/mL nalidixic acid. The colony was resuspended in 5 ml of sterile 0.1 % peptone by vortex mixing for 30 seconds using a vortex mixer. The $M17_{SNAR}$ cells present in the $M17_{SNAR}$ suspension were enumerated by performing serial 10-fold dilutions of the suspension into sterile 0.1 % peptone and plating 0.1 mL portions of each dilution onto Luria agar, Luria agar + 75 μg/mL nalidixic acid, VRBA, VRBA + 25 μg/mL nalidixic acid. The $M17_{SNAR}$ cell suspension was used to spike 10-1 dilutions of the fecal composite samples at two different concentrations by adding 0.1 mL of a 10-fold or 1000-fold dilution of $M17_{SNAR}$ suspension into the 10-1 dilution of independent fecal composite aliquots. The spiked 10-1 dilutions were then diluted by serial 10-fold dilutions into 0.1% peptone. 0.1mL aliquots of each dilution were then plated in duplicate onto the surface of Luria agar, Luria agar + 75 μg/mL nalidixic acid, VRBA, VRBA + 25 μg/mL nalidixic acid.

After the cells were spread onto the surface of the growth media, the plates were incubated for 36 hours at 37°C. The colonies were enumerated and averaged for each duplicate set of plates from the relevant dilutions. To confirm $M17_{SNAR}$, 10 colonies from each set of the VRBA + 25 μg/mL nalidixic acid plates of the relevant dilutions were picked with toothpicks and inoculated into 100 μL of Luria broth in 96-well culture plates. The plates were incubated for 16 hours at 37°C. From each culture, 50 μL of cells was then removed to a 96-well PCR plate and mixed with 50 μL of 20 mM Tris-0.2mM EDTA. The cells were then heated to 95 °C for 10 minutes in the thermocycler. From the heated cell suspensions, 2 μL was removed and distributed into a fresh 96-well PCR plate and 18 μL of PCR mix was added. The PCR mix consists of 1X PCR buffer (Takara), 250 mM dNTPs (Takara), 1.4 units of Taq DNA polymerase, 1uM of each primer (Contig 127 primers, above). The plate was then covered with a 96-well lid and placed into the thermocycler. Reactions were

heated to 95°C for 2.5 minutes followed by 40 cycles of 95°C for 30 seconds, 63 °C for 45 seconds, 72°C for 45 seconds. A final cycle of 72°C for 5 minutes was then conducted and the samples held at 4°C until ready for gel electrophoresis.

To the completed PCR reactions, 2 μL of loading dye (0.21% Bromphenol Blue, 0.21% Xylene cyanol, 50% glycerol) was added. A total of 15 μL of the reactions was then loaded onto a 0.8% agarose gel prepared in 1X TAE containing 1 ng/mL of ethidium bromide. The gel was then electrophoresed for 1.5 hours at 100 Volts/Cm. The electrophoresed PCR products were then visulalized by placing the gel onto a 302 nm UV lightbox and imaged with CCD camera.

Results

To measure the sensitivity and selectivity of the combined nalidixic acid resistance selection and Contig 127 PCR confirmation, experiments were performed with human fecal samples. A composite fecal sample was prepared from 10-gram samples of 100 human fecal samples. Samples of the composite were then mixed with measured quantities of the $M17_{SNAR}$ cells and subjected to serial dilution followed by plating of portions of each dilution onto VRBA + 25 μg/mL nalidixic acid as well as control media (VRBA, Luria agar, and Luria agar with 75 μg/mL nalidixic acid). After incubation, the number of colonies was averaged from duplicate plates of each dilution. As shown in Table 13, incorporation of 75 μg/mL nalidixic acid into Luria Agar was not selective for $M17_{SNAR}$ from fecal samples as this medium yielded nearly as many colonies as the Luria Agar alone. Therefore, nalidixic acid selection alone does not provide enough selectivity for $M17_{SNAR}$ detection. In contrast to the Lura agar, the VRBA + naldixic acid was entirely selective for $M17_{SNAR}$, as the fecal composite sample alone (without added $M17_{SNAR}$) yielded less than the detection limit of 10 CFU/ml lactose fermenting, nalidixic acid resistant colonies. Given that the fecal composite sample contained 1 X 10^4 lactose-fermenting CFU/ml on VRBA, the results argue that less than 1 in 10,000 coliform bacteria are capable of growth and lactose fermentation on VRBA + nalidixic acid. Samples in which $M17_{SNAR}$ cells had been introduced, however, did yield nalidixic acid-resistant, lactose-fermenting colonies in the VRBA + 25 μg/ml nalidixic acid media at the expected dilutions dilutions. Based on the number of input $M17_{SNAR}$

cells, the VRBA + 25 μg/mL nalidixic acid gave detection efficiencies of between 23% (for the 1.3 X 10^5 input sample) and 42% (for the 1.3 X 10^3 input sample). Therefore, the plating efficiency of the $M17_{SNAR}$ strain on VRBA + 25 μg/mL nalidixic acid is estimated at 32.5%. Combining the detection limit of < 10 CFU/mL from an undiluted fecal sample with the 32.5% plating efficiency of the $M17_{SNAR}$ on VRBA + 25 μg/ml nalidixic acid, a detection limit of <33 CFU/ml of $M17_{SNAR}$ in a fecal sample was determined. The results are summarized in Table 13 below.

To confirm that the colonies growing on the VRBA + nalidixic acid are only $M17_{SNAR}$, the Contig 127 PCR assay was performed on a total of 84 randomly chosen colonies from the VRBA + nalidixic acid plates derived from dilutions of samples containing $M17_{SNAR}$. To demonstrate the selectivity of the plating, 132 colonies from VRBA plates without antibiotic were also chosen randomly and tested with the Contig 127 PCR assay. Of the colonies chosen from VRBA plates, 48 were picked from plates where no $M17_{SNAR}$ had been added to the samples and 84 were chosen from the countable plates of dilutions from samples in which the $M17_{SNAR}$ had been introduced. As shown in Table 13, below, no Contig 127 PCR positive colonies were obtained from those tested from the VRBA plates derived from unspiked samples, as would be expected. Moreover, as was anticipated, both Contig 127-positive and Contig 127-negative colonies were detected on VRBA plates without antibiotics, with a higher ratio of positive to negative Contig 127 PCR positive colonies being obtained from the samples containing higher numbers of spiked $M17_{SNAR}$. When the colonies from VRBA + 25 μg/mL nalidixic acid were tested, only Contig 127-positive colonies were obtained, as would be expected. Therefore, the combination of selective plating on VRBA + nalidixic acid and Contig 127 PCR confirmation provides a highly sensitive and highly selective method for enumerating $M17_{SNAR}$ from fecal samples.

Table 13 - Enumeration of colonies from unspiked and spiked fecal samples.

	Input $M17_{SNAR}$	CFU/ml fecal sample	Contig 127 PCR confirmation[b]
LB	0	1.6 X 10^7	
LB	1.3 X 10^5	1.1 X 10^7	
LB	1.3 X 10^3	9.1 X 10^6	
LB + 75 μg/ml Nal	0	5.5 X 10^6	
LB + 75 μg/ml Nal	1.3 X 10^5	1.2 X 10^6	
LB + 75 μg/ml Nal	1.3 X 10^3	1.7 X 10^6	
VRBA	0	1 X 10^4	0/48
VRBA	1.3 X 10^3	1.1 X 10^6	40/48

	Input $M17_{SNAR}$	CFU/ml fecal sample	Contig 127 PCR confirmation[b]
VRBA	1.3×10^3	2.0×10^4	23/36
VRBA + 25μg/ml NaI	0	$>10^9$	
VRBA + 25μg/ml NaI	1.3×10^5	3.0×10^8	48/48
VRBA + 25μg/ml NaI	1.3×10^3	5.5×10^6	36/36

[a]No colonies present on the 10^{-4} dilution, the lowest dilution that was plated
[b]Number of colonies positive/number of colonies tested

EXAMPLE 6

Detection of $M17_{SNAR}$ in fecal samples from dogs prior to, during and following administration of the probiotic

The overall objective of this study was to develop a tool for the quantification of *E. coli* $M17_{SNAR}$ in human fecal samples and a method which would measure the degree and duration of shedding of the probiotic *E. coli* $M17_{SNAR}$ strain in fecal samples. Fecal samples collected from canines fed $M17_{SNAR}$ cultures during the course of a 14-day toxicology study (conducted by Ricerca Biosciences LLC, Concord, OH) were used to model this method. The approach used was to enumerate total coliforms and $M17_{SNAR}$ using selective plating on Violet Red Bile Agar (VRBA) and VRBA supplemented with 25 μg/mL nalidixic acid (as shown for spiked human fecal samples in Examples 5 above). Confirmation of $M17_{SNAR}$ was conducted on colonies growing on VRBA + 25 μg/mL nalidixic acid using the $M17_{SNAR}$-specific Contig 127 PCR assay.

Experimental Procedures

Differential plating of fecal samples - Fecal samples were collected during the course of a GLP dog toxicology study conducted by Ricerca Biosciences LLC, Concord, OH. Ten grams of each sample was mixed with 10 mL of sterile saline + 15 % glycerol in a sterile 50 mL conical tube. The slurries were then frozen at -80 °C and transported on dry ice. Each sample was logged into a master spreadsheet and subsequently stored at -80 °C until processing. For processing, the samples were thawed at room temperature for 1 hour and mixed vigorously by shaking for 30 seconds with an additional 10 mL of sterile 0.1 % peptone. The resulting slurry is referred to as the undiluted sample.

A 0.5 mL portion of the undiluted sample was removed with a P-1000 micropipettor and dispensed into 4.5 mL of sterile 0.1% peptone. The diluted sample was then serially diluted to a final dilution of 10^{-6}. For plating, 0.1 mL portions of the

undiluted sample and the 10^{-1} through 10^{-6} dilutions were plated in duplicate onto VRBA and VRBA + 25 μg/mL nalidixic acid. The plates were incubated for 36 hours at 37°C prior to enumerating colonies. Presumptive total coliforms were scored as the number of lactose-positive colonies on VRBA X reciprocal of the dilution. Presumptive $M17_{SNAR}$ were scored as the number of lactose-positive colonies on VRBA + 25 μg/mL nalidixic acid X reciprocal of the dilution.

PCR confirmation - To confirm presence of $M17_{SNAR}$, colonies were picked from VRBA or VRBA + 25 μg/mL nalidixic acid and tested using the Contig 127 PCR assay. Colonies to be tested were picked using sterile toothpicks and inoculated into 200 μL cultures of Luria Broth in sterile 96-well assay plates. Controls, including $M17_{SNAR}$ and DH5α, were also inoculated into the appropriate wells. The plates were then covered with a sterile plastic lid and incubated for 15 hours at 37°C. Following incubation, 50 μL of cells from each well was then transferred to the corresponding wells of a 96-well PCR plate and mixed with 50 μL of 20 mM Tris-0.2mM EDTA. The cells were then heated to 95 °C for 10 minutes in the thermocycler. From the heated cell suspensions, 2 μL was removed and distributed into a fresh 96-well PCR plate and 18 μL of PCR mix was added. The PCR mix consists of 1X PCR buffer (Takara), 250 mM dNTPs (Takara), 1.4 units of Taq DNA polymerase, 1uM of each primer. The plate was then covered with a 96-well lid and placed into the thermocycler. Reactions were heated to 95 °C for 2.5 minutes followed by 40 cycles of 95 °C for 30 seconds, 63 °C for 45 seconds, 72 °C for 45 seconds. A final cycle of 72 °C for 5 minutes was then conducted and the samples held at 4 °C until ready for gel electrophoresis.

To the completed PCR reactions, 2 μL of loading dye (0.21 % Bromphenol Blue, 0.21 % Xylene cyanol, 50 % glycerol) was added. A total of 15 μL of the reactions was then loaded onto a 0.8% agarose gel prepared in 1X TAE containing 1 ng/mL of ethidium bromide. The gel was then electrophoresed for 1.5 hours at 100 Volts/Cm. The electrophoresed PCR products were then visualized by placing the gel onto a 302 nm UV lightbox and imaged with CCD camera.

Results

Presumptive and confirmed detection of $M17_{SNAR}$ in canine fecal samples. Individual animals were dosed with $M17_{SNAR}$ daily for 14 days by gastric gavage. The animals were fed within 2 hours after administration of the $M17_{SNAR}$ dose. Fecal samples were collected daily beginning on day 0 and continuing through day 14 or day 28, depending on study group. Dosing was done at two different dose levels, 5 X 10^9 and 1 X 10^{13} cfu per dose, per day. For certain animals in the high dose group, dosing was stopped after day 14 and fecal samples continued to be collected for an additional 14 days. For example enumeration of $M17_{SNAR}$ in feces of selected animals from the high dose group, fecal samples from day 0 and days 1, 4, 7, 10, and 13 of the dosing period and days 16, 18, 20, 22, 25, and 28 post administration period were tested by differential plating on VRBA and VRBA + 25 μg/mL nalidixic acid.

Ten colonies from the highest dilutions of VRBA + 25 μg/mL nalidixic acid plates yielding countable colonies (30-300 colonies) were then subjected to Contig 127 PCR analysis to confirm the presence of $M17_{SNAR}$. Ten colonies were also tested from VRBA plates when the sample failed to yield any nalidixic acid-resistant colonies on even the undiluted sample. Table 14 shows the counts of presumptive total coliforms, presumptive $M17_{SNAR}$, and the results of the Contig 127 PCR assay. In each instance, only Contig 127-positive colonies were recovered from VRBA + nalidixic acid plates while no Contig 127-positive colonies were found among samples which failed to yield colonies on VRBA + nalidixic acid. Thus, the correlation between presumptive $M17_{SNAR}$ (growth of colonies on VRBA + nalidixic acid) was 1.0.

Table 14 - Presumptive total coliforms and $M17_{SNAR}$ from fecal samples of canines dosed with $M17_{SNAR}$.

Animal	Day	VRBA	VRBA + Nal	Contig 127 PCR (positives from 10 independent colonies)
		cfu (Log_{10})		
3028-3M	0	4.204	1.477	0
Male	1	7.176	6.792	10
	4	5.491	5.633	10
	7	6.380	6.204	10
	10	5.839	5.491	10
	13	6.968	6.544	10
	16	4.826	1.491	0
	18	5.643	1.491	0

Animal	Day	VRBA	VRBA + Nal	Contig 127 PCR (positives from 10 independent colonies)
		cfu (Log_{10})		
	20	6.146	1.491	0
	22	7.230	1.491	0
	25	4.342	1.491	0
	28	5.279	1.491	0
3029-3M	0	6.342	1.477	0
Male	1	6.176	4.863	10
	4	5.079	4.301	10
	7	6.903	6.079	10
	10	6.342	4.898	10
	13	7.255	6.477	10
	16	4.623	1.477	0
	18	6.505	1.477	0
	20	6.079	1.477	0
	22	6.041	1.477	0
	25	6.279	1.477	0
	28	6.771	1.477	0
3032-3F	0	4.869	1.477	0
Female	1	5.799	5.322	10
	4	6.114	5.672	10
	7	4.163	4.415	10
	10	4.763	4.556	10
	13	6.079	5.380	10
	16	3.176	2.079	0
	18	4.431	1.505	0
	20	4.204	1.505	0
	22	3.959	1.505	0
	25	4.919	1.505	0
	28	4.415	1.505	0
3033-3F	0	2.929	1.477	0
Female	1	5.462	5.398	10
	4	5.996	6.826	10
	7	7.415	7.146	10
	10	6.653	6.431	10
	13	5.940	6.633	10
	16	4.624	2.176	10
	18	5.959	1.518	0
	20	6.204	1.518	0
	22	7.415	1.518	0
	25	6.518	1.518	0
	28	6.613	1.518	0

Detectable shedding of M17$_{SNAR}$. The total coliform and total M17$_{SNAR}$ counts for the four animals are plotted in Figures 3a-d. The total coliform counts per

animal ranged between 10^4 and 10^7 CFU/g of feces. Shedding of $M17_{SNAR}$ was detectable in all four animals through day 13. Two animals continued to shed detectable levels of $M17_{SNAR}$ until day 16 (Figures 3c-d). Beyond day 16, $M17_{SNAR}$ could no longer be detected in the fecal samples. Thus, after dosing was stopped (day 14) the $M17_{SNAR}$ population was rapidly eliminated to levels below the detection limit of the assay ($10^{1.48}$ or 30 CFU/g).

During the dosing period, fluctuation was noted in both the total coliform counts and the $M17_{SNAR}$ counts. Whether this reflects statistical sampling error or other factors is unknown. After the period in which shedding was detectable, the total coliform levels generally reached the pre-administration levels, with the exception of animal 3033, in which the levels were approximately four-logs higher after dosing.

This study has shown that $M17_{SNAR}$ can be specifically detected in the feces of canines dosed with $M17_{SNAR}$. The $M17_{SNAR}$ does not appear to stably colonize the bowel as a member of the flora of these animals, and it is decreased to undetectable levels within 2-4 days after the dosing period. There was little effect of the $M17_{SNAR}$ on the total coliform population in terms of absolute numbers, although the data would not be able to measure any affects on diversity of the flora. Nonetheless, within 4-6 days after the dosing period, the total coliform levels are nearly as high, or higher than pre-administration levels.

Of note, there are some dose-response relationships that can be discerned during the first few days of the dosing period. It is likely that at these higher doses, the niche within the bowel that can be occupied by $M17_{SNAR}$ is quickly saturated. The assay method described in this study is sensitive and can detect appearance, duration and disappearance of $M17_{SNAR}$ in feces. Hence, this is an effective assay format that can be used to monitor colonization and elimination of *E. coli* $M17_{SNAR}$ during human clinical trials.

EXAMPLE 7

Genomic sequencing of $M17_{SNAR}$

A complete genome sequence of *Escherichia coli* $M17_{SNAR}$ was effected in order to identify genomic sequences unique to *E. coli* $M17_{SNAR}$. In order to accomplish this, whole genome shotgun sequencing strategy was used in which the

the genomic DNA is "peppered" with enough sequence 'reads' such that they overlap, and yield, when assembled, the complete sequence of the genome. An 8-fold coverage of the *Escherichia coli* M17$_{SNAR}$ genome was performed to obtain a comprehensive number of sequence 'reads' and a shotgun assembly was generated from the same. To complete the work, the following steps were taken sequentially,

1. Physical shearing of genomic DNA;

2. Construction of three separate libraries: one high titer small insert library, one 10 kb insert library and one 40 kb insert library;

3. Random shotgun sequencing to obtain approximately 8-fold sequence coverage of the genome; and

4. Assembly of the shotgun sequence data using highly specialized genome sequence assembly computer programs.

Experimental Procedures

Physical shearing of DNA -

E.coli M17$_{SNAR}$ genomic DNA was checked for molecular weight. Pulse field gel electrophoresis (PFGE) was run with a high molecular weight marker to determine the molecular weight of the sample (BioRad ChefMapper, Hercules, CA). The high molecular weight genomic DNA was then mechanically sheared via a Hydroshear™ device (Genemachines, San Carlos, CA) to the desired sizes of approximately 4.0kb, 10.0kb and 40.0kb. The Hydroshear™ machine provides a controlled and reproducible method available for generating random DNA fragments. This software-driven device (Hydroshear software v. 1.0.6a) uses hydrodynamic shearing forces to fragment DNA strands into designated sizes.

Library Construction

Experience has demonstrated the need for construction of several libraries including both, small and large-sized DNA inserts. The small-insert library is used to obtain an appropriate coverage of the genome and the large-insert library is used to obtain a 'scaffold' of the genome, which is used during the closure phase of the sequencing project. In other words, the ''reads'' from the small insert library provides the 'bulk' of the sequence information while the ''reads'' from the large-insert library help in assembling the sequence information in the correct order.

For the whole genome shotgun sequencing of *E.coli* $M17_{SNAR}$, three separate libraries containing 4, 10 and 40 kb DNA inserts were constructed using the sheared *Escherichia coli* $M17_{SNAR}$ DNA (Figure 4). The 3-4kb and 10.0kb fragments were cloned into the pAGEN vector system (Agencourt Bioscience Corp., Beverly, MA) for isolation and sequencing. To improve and verify assembly, a large insert fosmid library was generated with the 40 kb fragments using the CopyControl™ pCC1FOS™ vector system (Epicentre Technologies, Madison, WI). A fosmid is similar to a plasmid (circular DNA) but is capable of containing much larger sizes of DNA inserts, up to 50 kb, compared to about 10 kb in a plasmid. The large insert size (40 kb) of Fosmid libraries make them particularly attractive, since Fosmid clones can close small physical gaps with fewer walking steps, in comparison to small insert libraries and can therefore reduce redundant sequencing.

While constructing libraries it is essential that each recombinant clone contain a single genomic DNA insert. The presence of multiple inserts in a single clone would give rise to artifacts during genome assembly. In order to ensure this the randomly sheared DNA was used in construction of plasmid libraries using an adaptor based cloning method. Herein, the cloning vector was cleaved at a symmetrical pair of BstX1 sites to produce non-complementary 4-base overhangs as a result of which the vector fragment cannot recircularize. Mechanically sheared insert DNA was end-repaired and ligated (or 'joined') to an adaptor with an overhang complementary to the vector ends, but not to itself. The prepared inserts were then ligated or inserted into the vector. The use of non-self-complementary adaptors reduced background and substantially lowered the incidence of clone siblings.

Prior to proceeding with high throughput sequencing, the integrity of the transformed libraries was verified. A total of 768 clones were picked and subjected to bidirectional sequencing. A BLAST 2.2.10 (Basic Local Alignment Search Tool) analysis was performed on the 1,536 sequencing ''reads'' (www.ncbi.nlm.nih.gov/blast/). The BLAST program finds regions of local similarity between sequences. It compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches thereby providing valuable information about the possible identity and integrity of the 'query' sequences.

Random Shotgun Sequencing

Sequencing template preparation - The plasmids containing genomic DNA were isolated from library cultures using Solid Phase Reversible Immobilization technology (SPRI®) [Hawkins, TL., K.J. McKernan, L.B. Jacotot, J.B. MacKenzie, P.M. Richardson and E.S Lander. 1997. A magnetic attraction to high-throughput genomics. Science Vol. 276 (5320), 1887-1889]. High-copy plasmid templates (3-4kb and 10kb insert plasmids) were purified using SprintPrep™ SPRI protocol while the low-copy (40kb fosmid) templates were purified using a SPRI® protocol (Agencourt Bioscience Corp., Beverly, MA). The SPRI® (Solid Phase Reversible Immobilization) technology uses carboxylate-coated, iron-core, paramagnetic particles to capture DNA of a desired fragment length based on tuned buffering conditions. Once the desired DNA is captured on the particles, they can be magnetically concentrated and separated so that contaminants can be washed away. This procedure harvests plasmid DNA directly from lysed bacterial cultures by trapping both plasmid and genomic DNA to the functionalized bead particles and selectively eluting only the plasmid DNA.

Shotgun Sequencing - The DNA templates were sequenced in 384-well format using BigDye® Version 3.1 reactions on ABI3730 instruments (Applied Biosystems, Foster City, CA). The BigDye® Version 3.1 contains dye terminators labeled with novel high sensitivity dyes. This BigDye terminator chemistry involves a fluorescein donor dye linked to a dRhodamine acceptor dye and is 2-3 times brighter than standard dye terminators and also has narrower emission spectra giving less background noise. This provides an overall improvement of 4-5 times in sensitivity of the capillary analytical procedure. During sample preparation for sequencing, the DNA fragments are chemically labeled with these fluorescent dyes, which facilitate the detection and identification of the DNA.

In this procedure, labeled DNA samples are prepared in 96- or 384-well plates and placed on the ABI3730 Genetic Analyzer machine in which capillary electrophoresis is used in separating the mixture of DNA fragments according to their lengths, providing a profile of the separation and determining the order of the four deoxyribonucleotide bases. The DNA molecules from the samples are injected into thin, fuse-silica capillaries that have been filled with polymer. The DNA fragments

migrate towards the other end of the capillaries, with the shorter fragments moving faster than the longer fragments. As the fragments enter a detection cell, they move through the path of a laser beam which causes the dye on the fragments to fluoresce. This fluorescence is captured by a charge-coupled device (CCD) camera. The CCD camera coverts the fluorescence information into electronic information, which is then transferred to a computer workstation for processing by the 3700 data collection software to generate electropherograms which plot relative dye concentration against time for each of the dyes used to label the DNA fragments. The positions and shapes of the electropherogram are used to determine the base sequence of the DNA fragment. Thermal cycling for the sequencing reactions was performed using 384-well Thermocyclers (ABI, MJ Research, Hercules, CA). Sequencing reactions were purified using CleanSeq® dye-terminator removal kit from Agencourt Bioscience

Assembly

Once the shotgun sequencing phase is complete, the sequencing 'reads' generated from random subclones are assembled into contigs (contiguous sequences), followed by a directed, or finishing phase in which the assembly is inspected for correctness and for various kinds of data anomalies (such as contaminant 'reads', unremoved vector sequences, and chimeric or deleted 'reads'), additional data are collected to close gaps and resolve low quality regions, and editing is performed to correct assembly or base-calling errors.

Validation of sequence 'reads': All 'reads' obtained from the high-throughput sequencing were processed using Phred base calling software (version 0.020425c) and constantly monitored against quality metrics using the Phred Q20 (University of Washington). Phred is a base calling software developed at the University of Washington Genome Center which reads DNA sequence chromatogram files, analyzes peaks to call bases and assigns quality scores to each nucleotide (2). Phred Q20 calls bases with a Phred quality value of 20 or greater. A Phred score of 20 indicates the existence of one error in 100 bases. The quality scores for each run were monitored through Galaxy LIMS system, a state-of-the-art Oracle-based Laboratory Information Management System (LIMS) (Agencourt Bioscience Corp, Beverly, MA) with any substantial deviations from the normal range investigated immediately.

Assembly step - The assembly of the vast amount of sequence data generated into a few contigs (contiguous sequence) requires a complex computational process which is further complicated by three main considerations that need to be addressed,

1. quality scores of each sequence,
2. use of the generated sequence itself or its complement and
3. presence of repeated sequences whose mis-assembly needs to be avoided.

A range of computer software programs with complex algorithms, are now available to accomplish assembling an entire genome. Clusters of overlapping sequences are constructed and consensus sequences are deduced from these clusters. Paracel Genome Assembler™, version 2.6.2 (Paracel, Pasadena, CA), coupled with the LIMS system (Agencourt Bioscience Corp., Beverly, MA) were used to assemble the sequence data for this project. Paracel's scaffold viewer and Consed (version 13.0), a graphical tool for viewing and editing sequence assemblies (University of Washington) were used to finish the assembly [Gordon, D., C. Abajian and P. Green. 1998. Consed: a graphical tool for sequence finishing. Genome Res Vol. 8 (3), 195-202].

Results

Whole genome sequencing of *E.coli* $M17_{SNAR}$ was accomplished using the shotgun sequencing method. A multi-library strategy was utilized which helped to ameliorate the presence of repeat elements and other artifacts during assembly. Large insert libraries to increase the clone coverage and scaffolding of the genome assembly. In addition to a 3-4 kb-insert, high copy number plasmid library, a 10kb-insert plasmid and a 40kb-insert fosmid libraries were also constructed. Shotgun sequencing of the three libraries was performed and a total of 36,265,538 Phred 20 bases were generated to obtain approximately an 8-fold coverage of the genome. The sequence 'reads' were assembled into contiguous sequence blocks and subjected to a BLAST analysis for identification of sequences unique to *E.coli* $M17_{SNAR}$.

Pulse Field Gel Electrophoresis of *E.coli* $M17_{SNAR}$ genomic DNA

The whole-genome shotgun strategy involves randomly breaking DNA into segments of various sizes and cloning these fragments into vectors for sequencing. The success of this strategy is highly dependent on the quality and integrity of the

genomic DNA used as the starting material. A pulse field gel electrophoresis was run to check the quality of the genomic DNA and to determine if the DNA was high molecular weight.

During continuous field electrophoresis, DNA above 30-50 kb migrates with the same mobility regardless of size and is seen in a gel as a single large diffuse band. In the pulse field method the DNA is forced to change direction during electrophoresis and different sized fragments within this diffuse band begin to separate from each other. With each reorientation of the electric field relative to the gel, smaller sized DNA begins to move in the new direction more quickly than the larger DNA. Thus, the larger DNA lags behind providing a separation from the smaller DNA. Any low molecular weight or plasmid DNA can thus be identified.

High-throughput Sequencing

The high-throughput shotgun sequencing of *E.coli* $M17_{SNAR}$ resulted in 57,408 distinct high-quality 'reads' representing 36,265,538 bases with good quality scores, providing an approximately 8-fold coverage of the genome. The distribution of these sequences across the three different libraries constructed is shown in Table 15, below. The high copy standard 3-4 kb genome library offered the most cost efficient method to produce paired-end sequence coverage of the genome, while the 10 kb and Fosmid libraries provided larger physical links from paired-end 'reads' which are useful in ordering and orienting contiguous sequence 'blocks' in the assembly process.

Table 15 - Sequence 'reads' generated by the high-throughput sequencing of the three libraries

	Libraries		
	4kb insert	**10kb insert**	**Fosmid (40kb insert)**
Total 'reads'	27,648	13,728	16,032
Pass rate*	91.54%	83.75%	85.03%
Average Phred20 bases per 'read'**	725	763	670
Total Phred20 bases	18,349,009	8,772,363	9,144,166

* Pass rate: defined as a 'read' containing >100 cumulative Phred20 bases**

** Phred20 bases: Bases which receive a score of 20 or more when subjected to Phred analysis, a base-calling program developed at the University of Washington Genome Center.

Assembly

In the assembly phase, sequence data was vector and quality screened based on Phred quality score information. All the sequence 'reads' from the libraries were

first compared to each other. Identities between the sequences of different 'reads' were noted, and then used to align the sequences into contiguous stretches of sequence called contigs. The sequences of two different 'reads' of the same segment of DNA may not be identical because of the quality of the sequencing reaction analysis. Thus for each base in the contig it is usual to require that it is independently confirmed from multiple overlapping 'reads' from both directions.

Contig building software designed to take into account the "quality" of each base in a 'read' (where quality is a measure of the confidence the Phred software has that the base has been called correctly) were used. Any gaps, discrepancies or ambiguities in the sequence were also identified. Contigs were then ordered and linked together into larger supercontigs by using paired 'reads' lying in different contigs. A total of 464 contigs were assembled and the details are listed in Table 16, below. Whole genome assembly was thus performed using the Paracel Genome Assembler™, version 2.6:2, coupled with the Agencourt's LIMS system while, Paracel's scaffold viewer and Consed (version 13.0) were used to finish the assembly.

Table 16 - Summary of the sequence assembly

Contigs	Total #	Total length
Supercontigs	71	4420860
Contigs (≥2 kb)	313	4706396
Contigs (<2 kb)	80	na*
Total # of contigs	**464**	Na

* na, not applicable

Identification of sequences unique to E.coli M17$_{SNAR}$

To identify potential unique sequences, a BLAST analysis was effected to help determine sequences unique to *E. coli* M17$_{SNAR}$. BLAST, which stands for 'Basic Local Alignment Search Tool' (www.ncbi.nlm.nih.gov/BLAST/) is a program that finds regions of local similarity (alignment) between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. BLAST analysis allows for performing five distinct blast comparisons. For this study a blastn analysis was used which compares a given nucleotide sequence with other nucleotide sequences present in the database.

When the BLAST program finds an alignment to the 'query' sequence, it calls it a 'hit' and then scores the alignment based on the similarity between the two sequences using different statistical parameters. One of these is the Expect value (E), which is defined as a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. It decreases exponentially with the Score (S) that is assigned to a match between two sequences. Essentially, the E value describes the random background noise that exists for matches between sequences. For example, an E value of 1 assigned to a hit can be interpreted as meaning that in a database of the current size one might expect to see 1 match with a similar score simply by chance. This means that the lower the E-value, or the closer it is to "0" the more "significant" the match is.

When no sequence similarities are found in a BLAST analysis of a 'query' sequence against a particular database, it is recorded as "no hits found". This indicates that the query sequence is not found anywhere in the database making it a unique sequence.

Strategy devised to identify unique sequences:

All 464 total contigs were provided electronically as **.txt* files of the individual contig sequences in FASTA format (www.ncbi.nlm.nih.gov/blast/html/search.html). These files were used in a pairwise BLAST analysis of the entire contigs against the *E. coli* CFT073 genome sequence and subsequently to identify the non-aligning regions from the M17 contigs. These non-aligned sequences were then used in pairwise BLAST to identify any sequence homology against the *E. coli* MG1655 (K-12), *E. coli* EDL933 (O157:H7), and *E. coli* Sakai (O157:H7) genome sequences. Segments not showing significant alignment (sequence homology) to either the *E. coli* CFT073, K-12, or O157:H7 genomes were then used in a BLAST search against the entire nr NCBI database (www.ncbi.nlm.nih.gov/BLAST/).

In a parallel approach, BLAST analysis was effected with each of the 464 contigs first split up into 200bp (base pair) fragments and then individually blasted against two specialized databases. The first database constructed was an '*E. coli*' database, in which four strains of *E. coli* (www.ncbi.nlm.nih.gov/genomes/lproks.cgi) were included to create a database named NCBIrefseq_ecoli.dna *(Escherichia coli* strains

included were CFT073, K12, O157:H7 and O157:H7 EDL933). For the second database, all bacterial genomes present in the NCBI database on August 19, 2005 were consolidated to create a 'Bacterial' database and named NCBIrefseq_bacteria.dna. Next, each of the 200bp fragments was subjected to a blastn analysis against both consolidated NCBI databases of both *E. coli* and bacteria, described above. Sequences with 'no hits,' were identified to be unique sequences.

There were nine unique 200bp sequence fragments thus identified. All nine sequences had no hits against the '*E. coli*' database but 'hits' were found, although mostly with poor scores against the 'Bacteria' database.

Conclusion

Whole genome shotgun sequencing of *E. coli* $M17_{SNAR}$ was successfully completed to approximately an 8-fold coverage of the genome. Sequences unique to the organism were identified using BLAST analysis. The documented locations of the unique sequences found in the *E. coli* $M17_{SNAR}$ genome are listed in Table 17, below. For each of nine unique sequences, the following details are provided in Example 8,

The sequence, 200 base pairs in length.

BLAST 2.2.10 results

Against the *E. coli* database, NCBIrefseq_ecoli.dna: No hits were registered for any of the nine sequences.

Against the Bacterial database, NCBIrefseq_bacteria.dna: The top ten highest scoring hits are tabulated along with the respective 'E values'.

Based on 'blastn' analysis, none of the nine, 200 base pair long unique sequence fragments showed any homology to the consolidated *E. coli* database. There was homology found when these same sequence fragments were compared to a consolidated Bacterial database, even if the sequence homology was poor in most cases. For eight of the nine sequences, the sequence homology to bacteria other than *E. coli* varied from 7 to 14.5% base pair match (the percent homology for the top 'blastn' hit for each sequence is shown in Table 17 below.

Table 17 - Unique E. coli M17$_{SNAR}$ Sequences Identified by Shotgun Sequencing Approach

Appendix No.	Page No.	Sequence Identifier*	Non-*E. coli* Homology (%)	*E. coli* Homology (%)
1	13	081205.asm.C13_39801_40000	9.5	0.0
2	14	081205.asm.C120_38601_38800	10.0	0.0
3	15	081205.asm.C127_15201_15400	10.0	0.0
4	16	081205.asm.C166_1001_1200	14.5	0.0
5	17	081205.asm.C228_1401_1600	10.0	0.0
6	18	081205.asm.C250_3001_3200	90.0	0.0
7	19	081205.asm.C251_601_800	11.5	0.0
8	20	081205.asm.C274_2401_2600	13.5	0.0
9	21	081205.asm.C435_601_800	9.0	0.0

* Sequence identifiers indicate the origin of the fragments: Each of the 464 contigs assembled for this project were numbered C1 to C464. Once each contig was split into 200bp fragments for the BLAST analysis, each fragment was denoted by the contig number followed by the numbers of the base pairs included in the fragment.

For the unique sequence 081205.asm.C250_3001_3200, a 90% base pair match to *Salmonella enterica* subspecies was found.

In conclusion, the whole genome sequencing of *Escherichia coli* M17$_{SNAR}$ was completed and genomic sequences unique to *E. coli* M17$_{SNAR}$ have been successfully identified.

EXAMPLE 8

Sequence information

Example 8.1

Unique sequences identified with using BLAST analysis:

1. Sequence name: 081205.asm.C13_39801_40000 SEQ ID NO: 1
TTTTTTATGACCGAGTAAACAACAGGCTACGTCGCTCTTGGGTCATCGGT
TGTGCGTTTAATACACTTACAAGAGAACCTACCGTGGTGATGGGAAGAAA
CGGATTATCTTCTATTCCGCGTAGATCGCGCCCACAACGAGCGGCAATAT
CTTCGACCAGCTCTTCTAACTCGTCTGCATCGTTTACTAAATCCAGATCA

Blast Results:
A. Against *E.coli* Database:
Query= 081205.asm.C13_39801_40000 (200 letters)
Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

30B. Against Bacterial Database:

Table 18

Top 10 Sequences producing significant alignments:	**E Value**
Bacillus subtilis subsp. subtilis str. 168	0.47
Thermoanaerobacter tengcongensis MB4	1.8
Caulobacter crescentus CB15	1.8
Thermobifida fusca YX	7.3
Salmonella enterica subsp. enterica serovar	7.3
Silicibacter pomeroyi DSS-3 megaplasmid	7.3
Salmonella enterica subsp. enterica serovar	7.3
Rickettsia typhi str. Wilmington	7.3
Rhodopseudomonas palustris CGA009	7.3
Wolinella succinogenes DSM 1740	7.3

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8.2

2.SequenceName:081205.asm.C120_38601_38800 SEQ ID NO 2:
CCACAACATCCGACCAACGAAATCGTCCTGACGAGTGCTGTCCTGCGGGC
GGGCCAGAGCATGTTTGACGACTATAGCCTCTTTGCCCTGTATACCATCT
CGATAAGTATCTGCCCAAGCGTGCAACGTTTGATGCAGCACTGAATGATC
AGTGGAGCCCGCCGGTACGGTGTAAAGGATAGGAGTGACCCCTTTGGCCT

Blast Results:
A. Against *E.coli* Database
Query= 081205.asm.C120_38601_38800 nseq=468
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******
Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 19

Top 10 Sequences producing significant alignments:	E Value
Photobacterium profundum SS9 chromosome 1	0.12
Acinetobacter sp. ADP1	0.47
Chromobacterium violaceum ATCC 12472	0.47
Gluconobacter oxydans 621H	1.8
Pseudomonas syringae pv. phaseolicola 1448A	1.8
Burkholderia pseudomallei K96243	7.3
Burkholderia mallei ATCC 23344	7.3
Yersinia pseudotuberculosis IP 32953	7.3
Yersinia pestis biovar Medievalis str. 91001	7.3
Gloeobacter violaceus PCC 7421	7.3

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8.3

3. Sequence Name: 081205.asm.C127_15201_15400 SEQ ID NO: 3
ATTATGCAAGACCTTATATCAGCCTCCATCCGTACTGGACAAAATACCAT
TGTTCAAAAGTGGGGAAGATTCTATTCTCTTAGCATCGCTCGTTGGCTTG
CCACAGTATTAGCAGAACTGTCTGATATAGCTTCTCATAAATATGGAATA
ATTAGTTTTTATGGCCTTAGTGAACATTGTTGCAGTTATATAGTTGAAGA

Blast Results:

A. Against *E. coli* Database

Query= 081205.asm.C127_15201_15400 nscq=274
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 20

Top 10 Sequences producing significant alignments:	E Value
Wigglesworthia glossinidia	0.12
Zymomonas mobilis subsp. mobilis ZM4	7.3
Photobacterium profundum SS9	7.3
Prochlorococcus marinus subsp. pastoris str.	7.3
Chlorobium tepidum TLS	7.3
Thermotoga maritima MSB8	7.3

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8.4

4.SequenceName:081205.asm.C166_1001_1200 SEQ ID NO:
4CACGTACTAAGCTCTCATGTTTAACGTACTAAGCTCTCATGTTTAACGAA
CTAAACCCTCATGGCTAACGTACTAAGCTCTCATGGCTAACGTACTAAGC
TCTCATGTTTCACGTACTAAGCTCTCATGTTTGAACAATAAAATTAATAT
AAATCAGCAACTTAAATAGCCTCTAAGGTTTTAAGTTTTATAAGAAAAAA

Blast Results:
A. Against *E.coli* Database
Query= 081205.asm.C166_1001_1200 nseq=3
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 21

Top 10 Sequences producing significant alignments:	E Value
Pseudomonas fluorescens Pf-5	**0.008**
Ehrlichia ruminantium str. Welgevonden	0.47
Ehrlichia ruminantium str. Gardel	0.47
Ehrlichia ruminantium str. Welgevonden	0.47
Shewanella oneidensis MR-1	0.47
Rickettsia conorii str. Malish	0.47
Bacillus thuringiensis serovar konkukian str. 9	1.8
Photorhabdus luminescens subsp. laumondii TTO1	1.8
Bacteroides thetaiotaomicron VPI-5482	1.8
Streptococcus agalactiae NEM316	1.8

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8.5

5. Sequence Name: 081205.asm.C228_1401_1600 SEQ ID NO: 5
TTAGATAGTTTGTCTAAATAATTATGTTGCCATGCGAAGTATGCATGGCT
GCATGTCTGCCTTCCATTTAAAATGGGCTAAGACCTATAACCCTAAATAT
TATTCTTTATTATCTTCTTTACCACTTCGCACCATCCCGTTCGACTTGTT
GCGGTTGTACTTCGCCTGAAGCAGCTGGATTGGCGTCGGGCCATGCTCGG

Blast Results:
A. Against *E.coli* Database
Query= 081205.asm.C228_1401_1600 nseq=9
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 22

Top 10 Sequences producing significant alignments:	E Value
Lactobacillus acidophilus NCFM	0.12
Streptococcus pneumoniae R6	0.12
Streptococcus pneumoniae TIGR4	0.12
Mycoplasma mycoides subsp. mycoides SC str. PG1	0.47
Francisella tularensis subsp. tularensis Schu 4	1.8
Onion yellows phytoplasma OY-M	1.8
Gloeobacter violaceus PCC 7421	1.8
Erwinia carotovora subsp. atroseptica SCRI1043	1.8
Staphylococcus epidermidis RP62A	1.8
Pseudomonas aeruginosa PAO1	1.8

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8. 6

6. Sequence Name: 081205.asm.C250_3001_3200 SEQ ID NO: 6
AGCCGTCACGACCGCCGAAAGTGGCCGGGCGGCTGCGTACACGAAACTCT
CCTGGCTCAGGCATATCTTTCTGAGTGGACGGCAGCCCCAGCGTAAGCCT
GTTATCACGTAACTCCTTCAGTTGCCGCAGCGCTTCTTTGTGGTCATCCT
TCACCGTATCCGGGAGGTCACCTTCCGGGCGGCGGGCGTAGAGCCGGTAA

Blast Results:
A. Against *E.coli* Database
Query= 081205.asm.C250_3001_3200 nseq=30
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 23

Top 10 Sequences producing significant alignments:	**E Value**
Salmonella enterica subsp. enterica serovar	1e-93
Salmonella enterica subsp. enterica serovar	1e-93
Bradyrhizobium japonicum USDA 110	0.47
Mycobacterium leprae TN	0.47
Thermobifida fusca YX	1.8
Geobacillus kaustophilus HTA426	1.8
Symbiobacterium thermophilum IAM 14863	1.8
Pseudomonas syringae pv. phaseolicola 1448A	1.8
Rhodopseudomonas palustris CGA009	1.8
Pseudomonas fluorescens Pf-5	1.8

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8.7

7. Sequence Name: 081205.asm.C251_601_800 SEQ ID NO: 7
TGGATTGGTGGCAAACGCTGCCTTGCGATATTACATAAGACCACGAGACA
TATATTGCAGACCTATTCCTAGAGTCTGGCTAACCAGTGATCGCAACGCT
TCTTTCCCTTCAATTTTTATAGAGTCAGATATTCTGGCCCCCAACGGTTT
TTCCAGACTTCCAGGCGTAGCGTTTAATACCTCAAGACCTTTAGCCGTTA

Blast Results:
A. Against *E.coli* Database

Query= 081205.asm.C251_601_800 nseq=29
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 24

Top 10 Sequences producing significant alignments:	E Value
Bacillus clausii KSM-K16	**0.47**
Erwinia carotovora subsp. atroseptica SCRI1043	**0.47**
Gloeobacter violaceus PCC 7421	1.8
Methanocaldococcus jannaschii DSM 2661	1.8
Zymomonas mobilis subsp. mobilis ZM4	7.3
Bacillus cereus E33L	7.3
Parachlamydia sp. UWE25	7.3
Bdellovibrio bacteriovorus HD100	7.3
Lactobacillus johnsonii NCC 533	7.3
Rhodopirellula baltica SH 1	7.3

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8.8

8. Sequence Name: 081205.asm.C274_2401_2600 SEQ ID NO: 8
TTGTTATCATCGATCCTGATCTATGTCCTGCACCAGGGGAGTTTGTTGTC
GCCAAAAACGACGGTCACGAAGCTACATTTAAAAAATACCGTCCATTAGG
AATCGGCATCGACGACTTTGAATTAATCCCCCTAAATCCTGATTACCCTA
TTTTCGTAGTGCAGATATGAACTTACAGATCATAGGTGTAATGATCGAA

Blast Results:
A. Against *E. coli* Database

Query= 081205.asm.C274_2401_2600 nseq=88
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 25

Top 10 Sequences producing significant alignments:	E Value
Thermoplasma acidophilum DSM 1728	0.47
Silicibacter pomeroyi DSS-3	1.8
Desulfovibrio vulgaris subsp. vulgaris str.	1.8
Azoarcus sp. EbN1	7.3
Legionella pneumophila str. Paris	7.3
Propionibacterium acnes KPA171202	7.3
Bartonella henselae str. Houston-1	7.3
Gloeobacter violaceus PCC 7421	7.3
Synechococcus sp. WH 8102	7.3
Bacillus cereus ATCC 14579	7.3

Database: NCBIrefscq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

Example 8.9

9. Sequence Name: 081205.asm.C435_601_800 SEQ ID NO: 9
CGGCATTTAAAGCTGCCCTCACGAAGGTCTGTAATGGAATCTTCATTGTT
GAATCCCATGCCGACTATCCCCGAGGTTCCCGTAGTGTCCTGGTTATAAT
AATCCAGCATTGCTGTTCTTAACAGCGTTGCCCCTTGTGCGGACAGCAAC
TTACGTCCTGTATCAACTTTGCGCCCGTCGTCCCATCCACGGATAAACTC

Blast Results:
A. Against *E. coli* Database

Query= 081205.asm.C435_601_800 nseq=6
(200 letters)

Database: NCBIrefseq_ecoli.dna
6 sequences; 20,994,025 total letters

Searching..done

***** No hits found ******

Database: NCBIrefseq_ecoli.dna
Posted date: Aug 20, 2005 6:10 AM
Number of letters in database: 20,994,025
Number of sequences in database: 6

B. Against Bacterial Database

Table 26

Top 10 Sequences producing significant alignments:	E Value
Chlamydophila pneumoniae TW-183	1.8
Methanosarcina acetivorans C2A	1.8
Chlamydophila pneumoniae J138	1.8
Chlamydophila pneumoniae AR39	1.8
Chlamydophila pneumoniae CWL029	1.8
Photorhabdus luminescens subsp. laumondii TTO1	7.3
Salmonella enterica subsp. enterica serovar Typ	7.3
Salmonella enterica subsp. enterica serovar Typ	7.3

Database: NCBIrefseq_bacteria.dna
Posted date: Aug 19, 2005 10:25 AM
Number of letters in database: 788,089,043
Number of sequences in database: 413
BLASTN 2.2.10 [Oct-19-2004]

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention,

which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications and GenBank Accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application or GenBank Accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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(other references are cited in the document)

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WHAT IS CLAIMED IS:

1. A biologically pure culture of an *E. coli* M17 bacterial strain exhibiting nalidixic acid resistance.

2. A probiotic composition comprising, as an active ingredient, the bacterial strain of claim 1, and a carrier or diluent.

3. The composition of claim 2, comprising 10^3-10^{10} of bacterial cells of said bacterial strain per gram of the composition.

4. The composition of claim 2, wherein said carrier comprises a formulation for maintaining viability of said bacterial strain.

5. The composition of claim 4, wherein said formulation comprises a volatile fraction of a plant extract.

6. The composition of claim 2, further comprising an antifungal agent.

7. The composition of claim 2, further comprising an antibiotic.

8. The composition of claim 2, further comprising a probiotic microorganism selected from the group consisting of a yeast cell, a mold and a bacterial cell.

9. The composition of claim 2, wherein said carrier is a colonization carrier.

10. A food additive comprising as an active ingredient, the bacterial strain of claim 1, and a carrier suitable for human consumption.

11. The food additive of claim 9, wherein said colonization carrier is selected from the group consisting of a saccharide, a modified saccharide and a combination thereof.

12. A feed additive comprising as an active ingredient, the bacterial strain of claim 1, and a carrier suitable for animal consumption.

13. The feed additive of claim 12, wherein said carrier is selected from the group consisting of limestone, saccharides and wheat midds.

14. A foodstuff comprising the bacterial strain of claim 1.

15. The foodstuff of claim 14 being a milk product.

16. A method of treating a gastrointestinal disorder, the method comprising administering to a subject in need thereof a therapeutically effective amount of the bacterial strain of claim 1, thereby treating the gastrointestinal disorder.

17. The method of claim 16, wherein the gastrointestinal disorder is selected from the group consisting of pouchitis, ulcerative colitis, Crohn's disease, inflammatory bowel disease, celiac disease, small bowel bacterial overgrowth, gastroesophageal reflux disease, diarrhea, *Clostridium difficile* colitis and/or antibiotic associated diarrhea, irritable bowel syndrome, irritiable pouch syndrome, acute diarrhea, traveller's diarrhea, lactose intolerance, HIV-associated diarrhea, sucrose isomaltase deficiency, carcinogenesis, enteral feeding associated diarrhea, and disorders which are associated with enteropathogens, non-erosive esophageal reflux disease (NERD) and associated small bowel bacterial overgrowth, functional dyspesia, necrotizing enterocolitis, diabetes gastropathy and constipation.

18. The biologically pure culture, composition, food additive, feed additive, foodstuff, method of claims 1, 2, 10, 12, 15 and 16, wherein said bacterial strain comprises all the identifying characteristics of ATCC Deposit No. PTA-7295.

19. The biologically pure culture, composition, food additive, feed additive, foodstuff, method of claims 1, 2, 10, 12, 15 and 16, wherein said *E. coli* M17 is ATCC Deposit No. 202226 (DSM 12799).

20. The biologically pure culture, composition, food additive, feed additive, foodstuff, method of claims 1, 2, 10, 12, 15 and 16, wherein said *E. coli* M17 is selected from the group consisting of BU-239, BU-230-98, BU-230-01 and ATCC Deposit No. 202226 (DSM 12799).

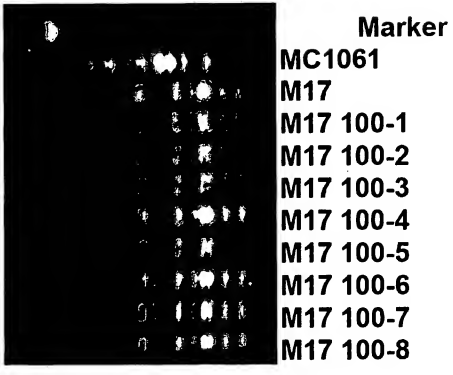
21. The biologically pure culture, composition, food additive, feed additive, foodstuff, method of claims 1, 2, 10, 12, 15 and 16, wherein said bacterial strain comprises a genomic nucleic acid sequence selected from the group consisting of SEQ ID NO: 1-9.

22. The biologically pure culture, composition, food additive, feed additive, foodstuff, method of claims 1, 2, 10, 12, 15 and 16, wherein said bacterial strain is capable of proliferating and colonizing in a mammalian gastrointestinal tract.

23. A method of detecting presence of the bacterial strain of claim 1 in a fecal sample, the method comprising detecting bacterial growth in the presence of nalidixic acid, thereby detecting presence of the bacterial strain in the fecal sample.

24. A biologically pure culture of an *E. coli* having all identifying characteristics of ATCC Deposit No. PTA-7295.

Fig. 1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MC1061
M17
M17 100-1
M17 100-2
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M17 100-5
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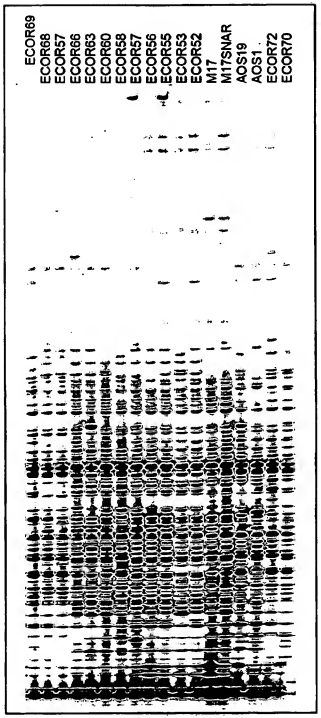
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ECOR66
ECOR63
ECOR60
ECOR58
ECOR57
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ECOR55
ECOR53
ECOR52
M17
M17SNAR
AOS19
AOS1
ECOR72
ECOR70

Fig. 2

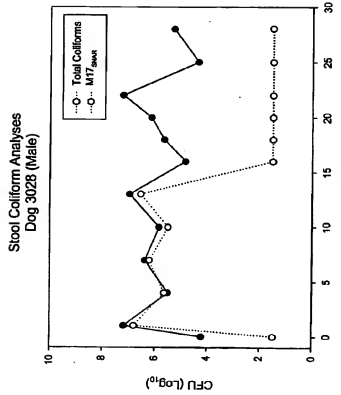
Stool Coliform Analyses
Dog 3028 (Male)
Total Coliforms
M17 SMAR
CFU (Log10)
Time (Days)
0
2
4
6
8
10
0
5
10
15
20
25
30

Fig. 3a

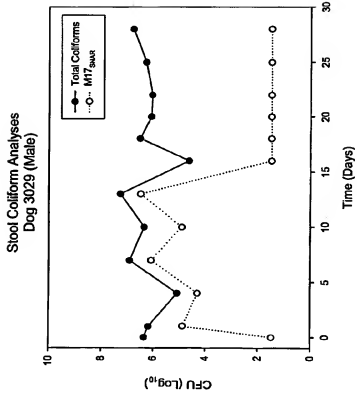
Stool Coliform Analyses
Dog 3029 (Male)
Total Coliforms
M17 BAAS
CFU (Log10)
Time (Days)
0
2
4
6
8
10
0
5
10
15
20
25
30

Fig. 3b

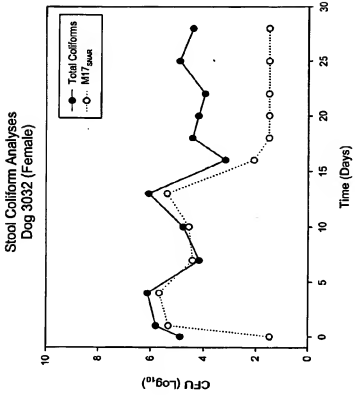
Stool Coliform Analyses
Dog 3032 (Female)
Total Coliforms
M17 plus
CFU (Log10)
Time (Days)
0
2
4
6
8
10
0
5
10
15
20
25
30

Fig. 3c

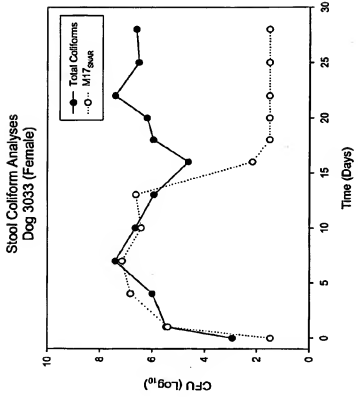
Stool Coliform Analyses
Dog 3033 (Female)
Total Coliforms
M17 SNR
CFU (Log10)
Time (Days)
0
2
4
6
8
10
0
5
10
15
20
25
30

Fig. 3d

E.Coli M17 DNA genome
4kb-
10kb-
40kb- DNA Fragments
4kb-insert library
10kb-insert library
Fosmid library
Sequence reads
Sequence of contigs
1. DNA Shearing
2. Library Construction
3. High-throughput Shotgun Sequencing
4. Assembly

Fig. 4